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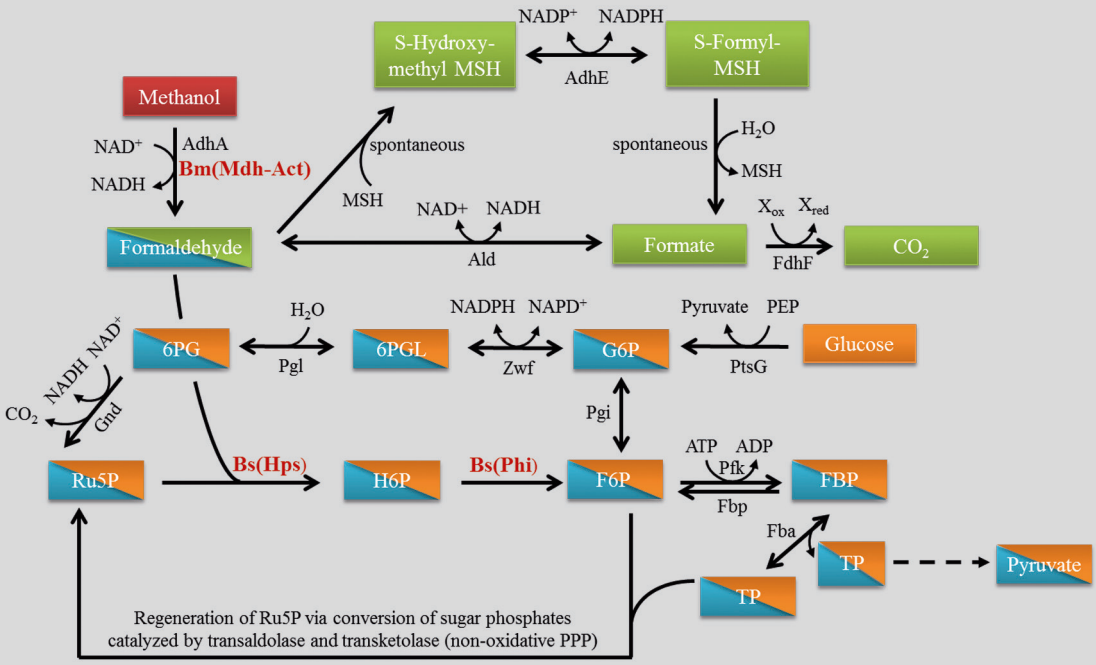
Sabrina Witthoff



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Content

Abbreviations

Abbreviations for Système International d'Unités (SI units) of measurement, chemical symbols for the elements and common abbreviations according to international standards, as for example listed in the author guidelines of the "Applied and Environmental Microbiology" journal are not listed in this section.

ATCC	American type culture collection	**FDH**	formate dehydrogenase
AT	aminotransferase	**FGH**	S-Formyl-glutathione hydrolase
BHI(S)	brain heart infusion (+ Sorbitol)	**FMH**	S-Formyl-MSH hydrolase
BGSC	Bacillus Genetic Stock Center	**FTIR**	Fourier transform infrared spectroscopy
CBB	Calvin-Benson-Bassham	**fw**	forward
CD	conserved domain	**F6P**	fructose-6-phosphate
CDW	cell dry weight	**GAP**	glyceraldehyde-3-phosphate
CH	cyclohydrolase	**GC**	gas chromatography
DAK	dihydroxyacetone kinase	**GFA**	GSH-formaldehyde-activating enzyme
DAS	dihydroxyacetone synthase	**GK**	glycerate kinase
DH	dehydrogenase	**GSH**	glutathione
DHase	dehydratase	**GST**	glutathione S-transferase
DHa	dehalogenase	**G6P**	glucose-6-phosphate
DHA(P)	dihydroxyacetone (phosphate)	**G6PDH**	glucose-6-phosphate dehydrogenase
DSMZ	Deutsche Sammlung für Mikroorganismen & Zellkulturen	**HPLC**	high-performance liquid chromatography
DMS	dimethylsufide	**HPR**	hydroxypyruvate reductase
DTT	dithiothreitol	**HPS**	3-hexulose-6-phosphate synthase
EMCP	ethylmalonyl-CoA pathway	**H_4F**	tetrahydrofolate
et al.	*et alii*	**H_4MPT**	tetrahydromethanopterin
E4P	erythrose-4-phosphate	**H6P**	hexulose-6-phosphate
FADH	formaldehyde dehydrogenase	**IPTG**	isopropyl-thio-β-D-galactopyranoside
FAE	formaldehyde activating enzyme	**Kan^R**	kanamycin resistance
FBA	FBP aldolase	**KDPG(A)**	2-keto-3-deoxy-6-phosphogluconate aldolase
FBP	fructose-1,6-bisphosphate	**LB**	Luria-Bertani broth
FBPase	fructose-1,6-bisphosphatase	**MADH**	methylamine dehydrogenase

MAOX	methylamine oxidase
MDH	methanol dehydrogenase
MeOX	methane oxidase
MNO	methanol:N,N′-dimethyl-4-nitrosoaniline oxidoreductases
MOX	methanol oxidase
MSA	methanesulfonic acid
MSH	mycothiol
NMPG	N-methylglutamate pathway
NMN	nicotinamide mononucleotide
OD_{600}	optical density at 600 nm
OAA	oxaloacetate
PEP	phoshoenolpyruvate
PEPC	PEP carboxylase
PGI	phosphoglucoisomerase
PHI	6-phospho-3-hexuloisomerase
PFK	phosphofructokinase
PPP	pentose phosphate pathway
PRI	phosphoriboisomerase
PQQ	pyrroloquinoline quinone
23PG	2-,3-phosphoglycerate
6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
6PGDHase	6-phosphogluconate dehydratase
6PGL	6-phosphoglucolactone
6PGLase	6-phosphoglucolactonase
RBS	ribosomal binding site
rev	reverse
Ri5P	ribose-5-phosphate
RPE	ribose-5-phosphate epimerase
RPI	ribose-5-phosphate isomerase
RuMP	ribulose monophosphate
Ru5P	ribulose-5-phosphate
SHMT	serine hydroxymethyl transferase
SBP	sedoheptulose-1,7-bisphosphate
SBPase	sedoheptulose bisphosphatase
S7P	sedoheptulose-7-phosphate
TAL	transaldolase
Tet^R	tetracycline resistance
TKT	transketolase
TPI	triosephosphate isomerase
XuMP	xylulose monophosphate
X5P	xylulose-5-phosphate
X_{ox}/X_{red}	unknown cofactor (oxidized/reduced)
ZTG	Zelltrockengewicht

Authors' contributions

***Corynebacterium glutamicum* harbours a molybdenum cofactor-dependent formate dehydrogenase which alleviates growth inhibition in the presence of formate.**

TP and SW designed the study and TP supervised it. The experimental work was performed by SW. The manuscript was written by TP and SW. LE and MB assisted in writing and reviewed the manuscript.

C_1 Metabolism in *Corynebacterium glutamicum*: an endogenous pathway for oxidation of methanol to carbon dioxide.

JM and SW designed the study and JM as well as MB supervised it. The experimental work performed by AM was supervised by SW. AM performed the construction of the Δ*adhA*, Δ*adhC*, Δ*adhE* and Δ*ald* mutants and SW constructed all other *C. glutamicum* strains. Enzyme assays were performed by AM. Global gene expression analysis as well as initial growth studies and ^{13}C-methanol-labeling experiments with *C. glutamicum* wild type as well as with Δ*adhA*, Δ*adhE* and Δ*adhC* mutants were performed by AM and SW. All other experimental work was performed by SW. The manuscript was written by JM, SW, and MB.

Metabolic engineering of *Corynebacterium glutamicum* for the metabolization of methanol.

JM and SW designed the study and JM as well as MB supervised it. The experimental work was performed by SW, and KS as well as SN assisted in analysis of the intracellular metabolite data. SW wrote the major part of the manuscript and JM as well as MB assisted in writing. SNo reviewed the manuscript.

AM: Alice Mühlroth, JM: Jan Marienhagen, KS: Katja Schmitz, LE: Lothar Eggeling, MB: Michael Bott, SN: Sebastian Niedenführ, SNo: Stephan Noack, SW: Sabrina Witthoff, TP: Tino Polen,

1 Summary

1.1 Summary English

Methanol is a pure and inexpensive raw material, which is mainly produced from fossil-fuel-based synthesis gas. Over the past years, new approaches were developed for its production from renewable carbon sources. In the chemical industry, methanol is already an important carbon feedstock, but it has found only limited application in biotechnology. This can predominantly be attributed to the inability of important microbial platform organisms to utilize this C_1 compound. With the aim to make methanol a suitable substrate for microbial production processes, the non-methylotrophic and industrially important amino acid-producing bacterium *Corynebacterium glutamicum* was engineered towards the utilization of methanol as auxiliary carbon source in a sugar-based medium.

Initial experiments on the response of *C. glutamicum* to methanol showed that this organism is able to oxidize methanol to CO_2 during the stationary phase with a rate of 0.83 ± 0.2 mM/h (2.8 ± 0.5 nmol min^{-1} mg CDW^{-1}) in glucose/methanol defined medium. Methanol oxidation was shown to be subject to carbon catabolite repression in the presence of glucose and to be dependent on the transcriptional regulator RamA. Global gene expression studies revealed that the alcohol dehydrogenase gene *adhA* as well as the aldehyde dehydrogenase gene *ald* were up-regulated in the presence of methanol. Analysis of a mutant lacking the *adhA* gene showed a 67% reduced methanol consumption rate (0.27 ± 0.05 mM/h), indicating that AdhA is mainly responsible for the oxidation of methanol to formaldehyde. The oxidation of formaldehyde to formate was found to be catalyzed predominantly by two enzymes, the acetaldehyde dehydrogenase Ald and the mycothiol-dependent formaldehyde dehydrogenase AdhE. A double mutant lacking *ald* and *adhE* was severely impaired in its ability to oxidize formaldehyde. The oxidation of formate to CO_2 is catalyzed by formate dehydrogenase (FDH). Deletion of *fdhF* (annotated as FDH) and *fdhD* (annotated as FDH accessory protein) in *C. glutamicum* abolished formate oxidation and resulted in an increased formate sensitivity. Growth studies with molybdenum and tungsten indicated that FdhF is a molybdenum-dependent enzyme. The electron acceptor of FdhF is not $NAD(P)^+$ and still unknown.

Heterologous expression of the methanol dehydrogenase gene (*mdh*) and the gene coding for the MDH activator protein Act (*act*) from *Bacillus methanolicus* increased the methanol oxidation rate of *C. glutamicum* from 0.83 ± 0.2 mM/h in the stationary phase to 1.7 ± 0.3 mM/h over the

course of the cultivation in glucose/methanol defined medium. The methanol oxidation rate in the stationary phase was 7.1 ± 0.4 nmol min^{-1} mg CDW^{-1}. Assimilation of formaldehyde was realized by implementing the key enzymes of the ribulose monophosphate pathway from *Bacillus subtilis*, 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI). These enzymes catalyze the condensation of formaldehyde with ribulose-5-phosphate to generate hexulose-6-phosphate and its isomerization to fructose-6-phosphate, respectively. Cultivation of the recombinant *C. glutamicum* strain expressing *mdh*, *act*, *hps* and *phi* in glucose/^{13}C-methanol defined medium led to labeling fractions of 3-10% in the m+1 mass isotopomers of selected intracellular metabolites, e.g. amino acids. The culture grew to a higher cell density in the presence of methanol than in medium without methanol and the final CDW was increased by 5% (8.4 ± 0.03 vs. 8.0 ± 0.05 mg/ml). Implementation of methanol oxidation and formaldehyde assimilation in the *C. glutamicum* Δ*ald*Δ*adhE* mutant, which was previously shown to be strongly impaired in its ability to oxidize formaldehyde to CO_2, increased the m+1 labeling of intracellular metabolites to 8-25%. However, the culture did not show a higher cell density compared to the culture grown without methanol in the medium. The engineered strains represent a promising starting point for amino acid production with *C. glutamicum* using methanol as auxiliary carbon source besides sugars.

1.2 Summary German

Methanol ist ein reiner und preiswerter Rohstoff der hauptsächlich durch die katalytische Umsetzung fossiler Energieträger hergestellt wird. In den letzten Jahren wurden zunehmend neue Verfahren entwickelt um Methanol basierend auf erneuerbaren Rohstoffen zu produzieren. Obwohl in der chemischen Industrie bereits als wichtiger Ausgangstoff für Synthesen eingesetzt, findet Methanol in der Biotechnologie bisher jedoch kaum Anwendung als Kohlenstoff-Quelle, da wichtige mikrobielle Plattformorganismen wie der Aminosäureproduzent *Corynebacterium glutamicum* nicht in der Lage sind C_1 Substrate zu verwerten. Um dies zu ändern, wurde *C. glutamicum* im Rahmen dieser Arbeit genetisch so modifiziert, dass Methanol als zusätzliche Kohlenstoffquelle während des Wachstums in zuckerbasiertem Medium genutzt werden kann.

Erste Wachstumsexperimente in Glucose- und Methanol-haltigem Medium haben gezeigt, dass *C. glutamicum* Methanol mit einer Rate von 0,83 ± 0,2 mM/h (2,8 ± 0,5 nmol min^{-1} mg ZTG^{-1}) in der stationären Phase zu CO_2 oxidieren kann. Zudem wurde festgestellt, dass die Oxidation von Methanol der Katabolitrepression in Anwesenheit von Glucose unterliegt und abhängig von dem Transkriptionsregulator RamA ist. Mittels globaler Genexpressionsanalysen wurde deutlich, dass die Gene der Alkoholdehydrogenase AdhA und die der Aldehyd-Dehydrogenase Ald (*adhA* and *ald*) in Anwesenheit von Methanol hochreguliert waren. Analyse einer *C. glutamicum* Mutante mit fehlendem *adhA* Gen zeigte, dass diese eine um 67% reduzierte Methanol-Oxidationsrate aufwies (0,27 ± 0,05 mM/h). Dies deutet darauf hin, dass die AdhA in *C. glutamicum* hauptverantwortlich für die Oxidation von Methanol zu Formaldehyd ist. Die Oxidation von Formaldehyd zu Formiat wird in erster Linie durch zwei Enzyme katalysiert: Die Acetaldehyd-Dehydrogenase Ald und die Mycothiol-abhängige Formaldehyd-Dehydrogenase AdhE. Eine daraufhin konstruierte Δ*ald*Δ*adhE* Doppelmutante konnte kaum noch Formaldehyd oxidieren. Die Oxidation von Formiat zu CO_2 wird durch eine Formiat-Dehydrogenase (FDH) katalysiert. Die Deletion von *fdhF* (annotiert als FDH) und *fdhD* (annotiert als FDH-Helferprotein) in *C. glutamicum* führte zu einer erhöhten Sensitivität gegenüber Formiat und zu einem Verlust der Fähigkeit Formiat zu oxidieren. Wachstumsstudien mit Molybdän und Wolfram deuten darauf, dass FdhF ein Molybdän-abhängiges Enzym ist. Der Elektronenakzeptor der FdhF konnte bisher nicht identifiziert werden.

Die heterologe Expression von *mdh* (Gen der Methanol-Dehydrogenase) und *act* (Gen des MDH Aktivatorproteins Act) aus *Bacillus methanolicus* führte zu einem Anstieg der Methanol-Oxidation von 0,83 ± 0,2 mM/h in der stationären Phase auf 1,7 ± 0,3 mM/h über den Verlauf der gesamten Kultivierungsdauer. Die Methanoloxidationsrate in der stationären Phase lag bei 7,1 ± 0,4 nmol min^{-1} mg ZTG^{-1}. Zur Assimilation von Formaldehyd wurden die Schlüsselenzyme des Ribulose-Monophosphat Weges aus *Bacillus subtilis* in *C. glutamicum* eingebracht, die 3-Hexulose-6-Phosphat Synthase (HPS) sowie die 6-Phospho-3-Hexulose Isomerase. Diese Enzyme katalysieren die Kondensation von Formaldehyd mit Ribulose-5-Phosphat zur Bildung von Hexulose-6-Phosphat beziehungsweise dessen Isomerisierung zu Fructose-6-Phosphat. Kultivierung des rekombinanten *C. glutamicum* Stammes, der *mdh*, *act*, *hps* und *phi* exprimiert, in ^{13}C-Methanol/Glucose-haltigem Medium führe zu einer 3-10 %igen m+1 Markierung von ausgewählten intrazellulären Metaboliten, wie z.B. Aminosäuren. In Anwesenheit von Methanol wuchs die Kultur zu einer höheren Zelldichte als in Medium ohne Methanol und das Zelltrockengewicht war um 5% erhöht (8,4 ± 0,03 vs. 8,0 ± 0,05 mg/ml). Implementierung der Module zur Methanol-Oxidation und Formaldehyd-Assimilation in die *C. glutamicum* Δ*ald*Δ*adhE* Mutante, welche in ihrer Fähigkeit Formaldehyd zu oxidieren sehr stark eingeschränkt ist, resultierte in einer Erhöhung der m+1 Markierung der intrazellulären Metabolite auf 8-25%. Jedoch wuchs die Kultur nicht zu einer höheren Zelldichte heran als die Kultur welche ohne Methanol im Medium angezogen wurde. Die Ergebnisse dieser Arbeit sind ein vielversprechender Ausgangspunkt für die Produktion von Aminosäuren mit *C. glutamicum* auf der Basis von Methanol als zusätzliche Kohlenstoffquelle neben Zuckern.

2 Introduction

The biotechnological production of amino acids, alcohols, organic acids, fine chemicals or proteins depends mainly on sugars as carbon source. However, the availability and price of sugar is dependent from seasonal variations and weather conditions as well as on price regulations and import limitations imposed on agricultural products. This leads to strong fluctuations in the sugar price and due to the increasing world population and loss of arable land it is even expected to rise in the coming decades. Thus, the demand for an alternative carbon source arises (Schrader *et al.*, 2009). Methanol is a pure and inexpensive raw material and in the chemical and fuel industry, it is already an important carbon feedstock. In 2012/2013 about 100 million metric tons (90 billion liters) were produced per year by over 90 methanol production plants worldwide (Methanol Institute, www.methanol.org, August 2014). At present, methanol is produced mainly from synthesis gas (a mixture of CO and H_2), which is obtained by catalytic reforming of coal or natural gas. A newly developed method "oxidative bi-reforming" allows direct oxygenation of methane to methanol in an economic and energetically efficient process (Olah *et al.*, 2013a; Olah *et al.*, 2013b). However, to be independent from fossil raw materials, production of renewable methanol was focused over the past years. Municipal waste, industrial waste or biomass can be gasified to produce synthesis gas for catalytic conversion to methanol. These pathways are already commercialized, e.g., the companies Enerkem (Canada) and BioMCN (Netherlands) produce renewable methanol based on municipal waste and glycerol, respectively and the company VärmlandsMethanol AB (Sweden) is in the process of building a biomass-to-methanol plant (Law *et al.*, 2013). CO_2 can also be converted into methanol by hydrogenation over homogenous and heterogeneous catalysts. The latter technology is already being used commercially in Iceland by Carbon Recycling International (CRI) (Olah, 2013; Wesselbaum *et al.*, 2012). Availability and market price of methanol (August 2014: Ø 450 USD/MT, http://www.methanex.com) raises the question if this C_1-compound could serve as alternative carbon source for microbial production processes (Brautaset *et al.*, 2007; Koopman *et al.*, 2009).

In the 1970's, the Phillips Petroleum Company developed a process for growing *Pichia pastoris* in continuous cultures at high cell densities to obtain single cell protein (SCP) from methanol. However, the use of methanol became unattractive because of the oil crisis in these years (Cos *et*

al., 2006). Nowadays, methanol is mainly used as auxiliary substrate in glycerol-based fermentation processes to induce heterologous gene expression during production of recombinant glycosylated proteins like insulin or several hepatitis B vaccines with the methylotrophic yeasts *P. pastoris* and *Hansenula polymorpha* (Cereghino & Cregg, 2000; Gellissen *et al.*, 2005). Production of other interesting compounds employing biotechnological processes were described for some methylotrophic microorganisms: Production of 9.5 g/L (poly)hydroxybutyrate was described for *Methylobacterium extorquens* DSMZ1340 (Khosravi-Darani *et al.*, 2013; Mokhtari-Hosseini *et al.*, 2009), and 59 g/L L-glutamate were produced by *Bacillus methanolicus* MGA3 (wild type) during fed-batch processes at 50°C. The high flux towards glutamate is explained with the high activity of the citrate synthase leading to the efficient conversion of cellular carbon to 2-oxoglutarate, with the high expression of the glutamate synthase in presence of ammonia and the low 2-oxoglutarate dehydrogenase activity. In addition, efficient secretion of glutamate is obtained during cultivation at high temperatures (Brautaset *et al.*, 2003). The *B. methanolicus* threonine and methionine auxotrophic and homoserine dehydrogenase defective mutant NOA2#13A52-8A66 produced even up to 65 g/L L-lysine under optimized fed-batch methanol fermentations. This strain was constructed by multiple cycles of classical mutagenesis (Brautaset *et al.*, 2010). However, the yield of methanol-based amino acid production is yet not economically feasible to establish production processes at industrial scale. The maximum L-lysine productivity obtained by *B. methanolicus* based on methanol as carbon source was only 1.6 g/L/h (L-lysine yield: 65 g/L) (Brautaset *et al.*, 2010), whereas the maximum L-lysine productivity obtained by *C. glutamicum* based on glucose as carbon source was 4.0 g/L/h (L-lysine yield: 120 g/L) (Becker *et al.*, 2011).

2.1 Methylotrophic metabolism

Methylotrophs can generate biomass and energy from reduced C_1 carbon substrates such as methanol, methane, methylated amines, halogenated methanes, and methylated sulfur species. These compounds occur abundantly in nature, e.g. methanol is formed during mineralization processes, mostly from degradation of methylesters and methylethers, being constitutes of pectin and lignin (Kloosterman *et al.*, 2002). Methylotrophy can be found in the genera of α-, β-, and γ-proteobacteria as well as within Gram-positive bacteria and yeasts (Chistoserdova, 2011).

These organisms are widespread in nature and play an important role in the global carbon cycle while comprising the principal biological sink for methane and other methylated greenhouse gases (Lidstrom, 2006; Yurimoto *et al.*, 2005). The aerobic methylotrophic metabolism can be divided into three parts: (i) oxidation or degradation of methyl-containing C_1 compounds, (ii) oxidation of formaldehyde or methyl-/methylene-H_4F and (iii) assimilation of formaldehyde, CO_2 and/or methylene-H_4F (FIG 1). The occurrence and combination of these pathways for oxidation and assimilation of C_1 compounds is highly diverse in the microbial world. A detailed overview of the modularity of methylotrophy is given in the review of Chistoserdova (2011). Pathways for anaerobic methylotrophy can be found in Clostridia (Adamse & Velzeboer, 1982) and Archaea (Costa & Leigh, 2014), but will not be discussed here.

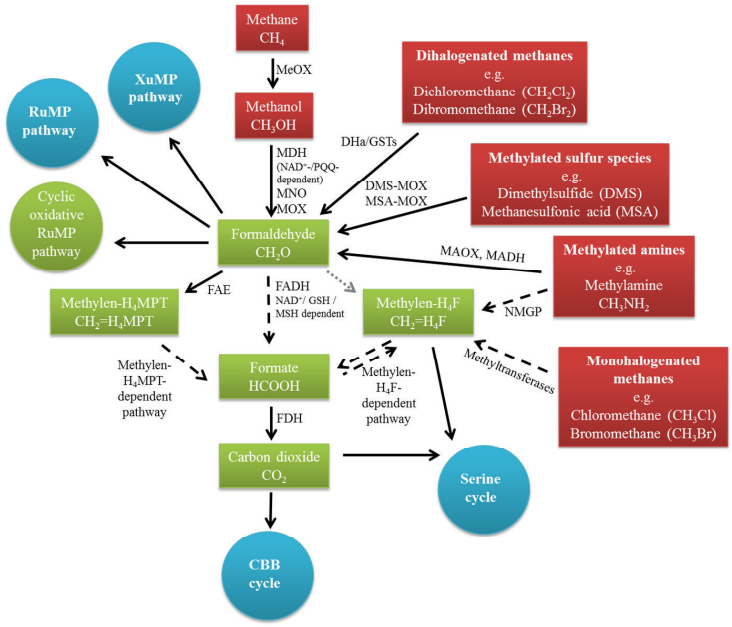

FIG 1 Simplified diagram of pathways attributed to methylotrophic metabolism. Methyl-containing C_1 compounds that are subject to oxidation/degradation are shown in red, formaldehyde/methylene-H_4F oxidation pathways in green and C_1 assimilation pathways in blue. Dashed lines represent reactions catalyzed by a series of reactions and the grey dotted line indicates a non-enzymatic reaction. Abbreviations: CBB, Calvin-Benson-Bassham; DHa/GSTs, dehalogenase/glutathione *S*-transferase; DMS/MSA MOX, dimethylsulfide/methanesulfonic acid monooxygenase; FADH, formaldehyde dehydrogenase; FAE, formaldehyde activating enzyme; FDH, formate dehydrogenase; H_4F, tetrahydrofolate; H_4MPT, tetrahydromethanopterin; MAOX/MADH, methylamine oxidase and methylamine dehydrogenase; MDH, methanol dehydrogenase; MeOX, methane oxidase; MNO, methanol:N,N′-dimethyl-4-nitrosoaniline oxidoreductase; MOX, methanol oxidase; NMGP, N-methylglutamate pathway; PQQ,, Pyrroloquinolin quinone; RuMP, ribulose monophosphate; XuMP, xylulose-monophosphate. Adapted from Chistoserdova (2011).

2.1.1 Oxidation of methylated substrates

The oxidation of methylated substrates is the first step in methylotrophic metabolism. Methanotrophs are a subgroup of the methylotrophic bacteria and have the ability to grow on methane as sole carbon and energy source. The aerobic oxidation of methane is catalyzed by either soluble or membrane-bound methane oxidases (MeOX), but the membrane-bound MeOXs are more widespread among methanotrophs (Chistoserdova, 2011; Hanson & Hanson, 1996). Methylated amines, halogenated methanes, and methylated sulfur species can either be directly oxidized to formaldehyde, or can be degraded to methylene-H_4F by a series of reactions (FIG 1).

Microbial evolution has led to four different enzymatic strategies for the oxidation of methanol to formaldehyde in methylotrophic organisms: quinoprotein methanol dehydrogenases (PQQ-MDHs), NAD^+-dependent MDHs, methanol:N,N'-dimethyl-4-nitrosoaniline oxidoreductases (MNOs) as well as methanol oxidases (MOXs). FAD^+-dependent MOXs belong to the short chain alcohol oxidases and oxidize methanol with concomitant reduction of oxygen to hydrogen peroxide in the peroxisomes of methylotrophic yeast and fungi (Goswami *et al.*, 2013; van der Klei *et al.*, 1991). Gram-negative methylotrophic bacteria, such as *M. extorquens,* use PQQ-dependent MDHs to oxidize methanol in the periplasm. Electrons obtained during oxidation of PQQ are first transferred to a specific cytochrome *c*, and finally to the terminal oxidase (Goodwin & Anthony, 1998; Nakagawa *et al.*, 2012). MNOs with tightly bound NADPH cofactor were found e.g. in *Amycolatopsis* and *Mycobacterium* (Bystrykh *et al.*, 1993) and Gram-positive thermotolerant *Bacillus* strains usually contain NAD^+-dependent cytoplasmic methanol dehydrogenases (Arfman *et al.*, 1989). The NAD^+-dependent MDHs are decameric proteins and each subunit contains one Zn^{2+}-ion and one or two Mg^{2+}-ions and additionally non-covalently bound NAD(H) (Arfman *et al.*, 1997; Vonck *et al.*, 1991). The MDH-bound NAD^+ cofactor serves as primary electron acceptor, and the exogenous NAD^+ coenzyme is responsible for the re-oxidation of the MDH-bound NADH. This reaction follows a ping-pong mechanism (Arfman *et al.*, 1997). The activity is strongly stimulated by an activator protein Act, which belongs to the family of "nucleotide diphosphate linked to some other moiety X" (Nudix) hydrolases. Act catalyzes the hydrolytically removal of the nicotinamide mononucleotide (NMN(H)) moiety from the NAD(H) cofactor resulting in diffusion of this moiety out of the cofactor binding site. Thus, the ping-pong reaction type is changed into a cofactor-independent

ternary complex mechanism (Arfman *et al.*, 1991; Arfman *et al.*, 1997; Kloosterman *et al.*, 2002). Both reaction mechanisms and the transition between these are visualized in FIG 2.

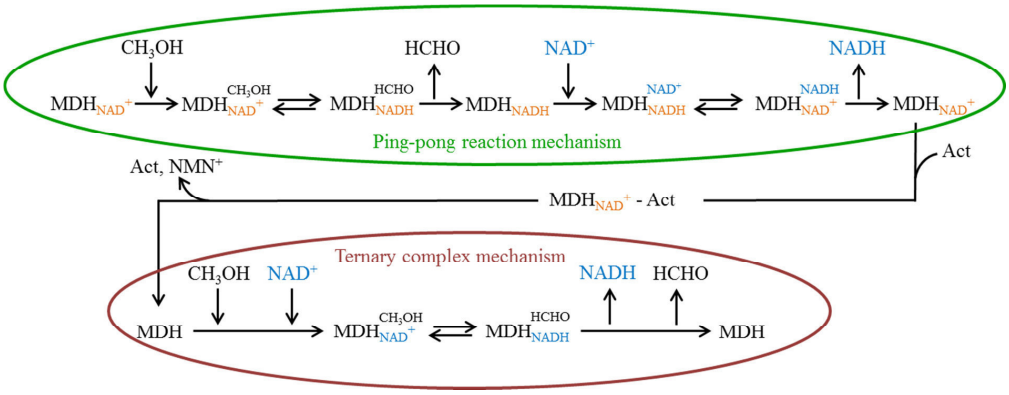

FIG 2 Reaction mechanisms of the methanol dehydrogenase (MDH) in the presence and absence of the activator protein Act. In absence of Act, the NAD(H) cofactor (orange) functions as a temporary electron deposit, and the reaction proceeds via a ping-pong reaction mechanism, in which the exogenous NAD^+ coenzyme (blue) is responsible for the re-oxidation of the NADH cofactor. Act catalyzes the cleavage of the NAD(H) cofactor which results in diffusion of the NMN(H) moiety out of the cofactor binding site, and the reaction proceeds via a ternary complex mechanism (based on Kloosterman *et al.*, 2002).

2.1.2 Oxidation of formaldehyde to CO_2

During methylotrophic growth, the oxidation of formaldehyde is important for the generation of energy in form of NADH, but is also indispensable for prevention of a toxic accumulation of formaldehyde. The toxicity of formaldehyde is due to non-enzymatic reactions with biological macromolecules such as proteins and DNA, leading to irreversible alkylations and cross-linkages (Bolt, 1987; Chen *et al.*, 2013; Heck *et al.*, 1990). In methylotrophic metabolism, formaldehyde is generated via oxidation of methylated substrates; however, virtually all organisms have to cope with formaldehyde as it is a byproduct of numerous environmental processes and various cellular demethylation and oxidation reactions, such as bacterial degradation of methoxylated lignin monomers (e.g. vanillin or vanillic acid) (Mitsui *et al.*, 2003). Thus, pathways for formaldehyde detoxification are widely distributed and not limited to methylotrophs. Formaldehyde oxidation proceeds either via linear pathways or via the cyclic dissimilatory ribulose monophosphate (RuMP) pathway. The cyclic RuMP pathway proceeds like the assimilatory RuMP pathway (FIG 4) with the participation of 6-phosphogluconate

dehydrogenase catalyzing the oxidation of 6-phosphogluconate to ribulose-5-phosphate along with release of CO_2. This cyclic pathway can mainly be found in Gram-positive methylotrophs assimilating formaldehyde via the RuMP cycle (Lidstrom, 2006).

The linear oxidative pathways can be cofactor-independent or they involve trapping of formaldehyde by cofactors, such as glutathione, mycothiol, tetrahydrofolate, or tetrahydromethanopterin (H_4MPT) (Vorholt, 2002; Yurimoto *et al.*, 2005). An overview of the linear dissimilatory pathways is given in FIG 3. Most methylotrophic organisms possess more than one pathway for formaldehyde oxidation, which contribute to the detoxification of formaldehyde and/or in the generation of energy (Vorholt, 2002).

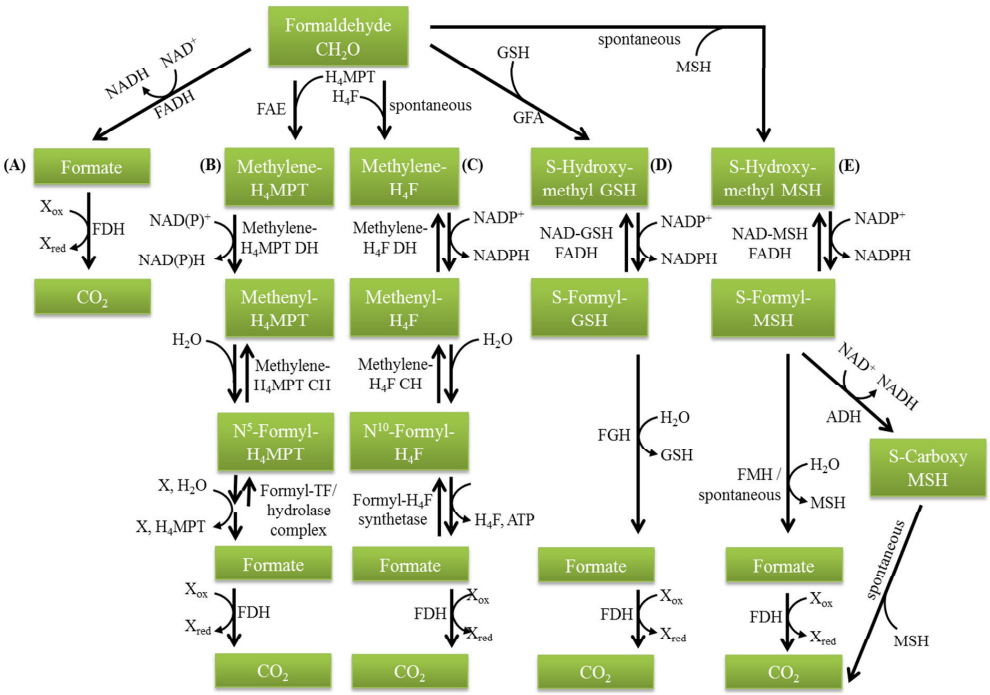

FIG 3 Linear pathways for formaldehyde oxidation via (A) the cofactor-independent pathways, or via (B) tetrahydromethanopterin (H_4MPT)-, (C) tetrahydrofolate (H_4F)-, (D) glutathione (GSH)- or (E) mycothiol (MSH)-dependent pathways. Abbreviations: ADH, aldehyde dehydrogenase; CH, cyclohydrolase; DH, dehydrogenase; FAE, formaldehyde-activating enzyme; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; FGH, S-Formyl-glutathione hydrolase; FMH, S-Formyl-MSH hydrolase; GFA, GSH-dependent formaldehyde-activating enzyme; TF, transferase; X_{ox}/X_{red}, different possible cofactors in their oxidized or reduced form. Adapted from Lidstrom (2006) and Vorholt (2002).

The simple oxidation of formaldehyde to formate via NAD^+-dependent and cofactor-independent formaldehyde dehydrogenases (FADH) (FIG 3A) is hardly found in methylotrophs. Instead this route seems to be mostly taken by non-methylotrophs, such as *Pseudonomas putida* (Chistoserdova, 2011; Tanaka *et al.*, 2003).

The H_4MPT-linked pathway for formaldehyde oxidation (FIG 3B) is broadly distributed throughout nature as it can be found in all Gram-negative methylotrophs employing the serine cycle or the RuMP pathway for formaldehyde assimilation (Vorholt *et al.*, 1999). This pathway is inducible by methanol, which indicates its major role in methylotrophic growth (Vorholt *et al.*, 1998). H_4MPT-dependent formaldehyde oxidation is initiated by the condensation of H_4MPT and formaldehyde which is accelerated by the formaldehyde-activating enzyme Fae. The generated N^5,N^{10}-methylene-H_4MPT is subsequently oxidized to N^5,N^{10}-methenyl-H_4MPT by two pyridine nucleotide-dependent dehydrogenases. Further conversion is performed via a methenyl-H_4MPT cyclohydrolase and the formyltransferase/hydrolase complex to formate (Hagemeier *et al.*, 2000; Pomper *et al.*, 1999; Vorholt *et al.*, 1998; Vorholt, 2002).

The H_4F-dependent formaldehyde oxidation (FIG 3C) is found in many methylotrophs but corresponding genes are generally expressed only at a low basal level (Vorholt, 2002). It is initiated via spontaneous condensation of formaldehyde and H_4F to N^5,N^{10}-methylene-H_4F. Typically, this pathway involves a formyl-H_4F synthetase and FolD, a bifunctional enzyme possessing methylene-H_4F dehydrogenase and methenyl-H_4F cyclohydrolase activity (Maden, 2000). The latter two reactions can also be catalyzed by two separate enzymes, as shown for *M. extorquens* (Pomper *et al.*, 1999; Vorholt *et al.*, 1998).

Thiol-linked formaldehyde oxidation is the most widespread pathway for formaldehyde conversion, since it is not only found in methylotrophic bacteria, but also in yeasts and in non-methylotrophic organisms where this route is involved in formaldehyde detoxification. Glutathione-dependent oxidation (FIG 3D) can be mainly found in Gram-negative autotrophic methylotrophs (Vorholt, 2002). The formaldehyde conversion starts with spontaneous condensation of formaldehyde and glutathione (GSH) leading to S-hydroxy-methylglutathione. This reaction was found to be accelerated by a GSH-dependent formaldehyde-activating enzyme (Gfa) (Goenrich *et al.*, 2002). NAD(H)-GSH-dependent FADHs are induced under methylotrophic conditions (Barber & Donohue, 1998; Van Ophem & Duine, 1994) and oxidize S-

hydroxy-methylgluthatione to S-formyl-glutathione, which is further converted to glutathione and formate by a S-formyl-glutathione hydrolase (FGH) (Vorholt, 2002). Some Gram-positive bacteria contain mycothiol instead of glutathione and the main pathway for formaldehyde oxidation involves a mycothiol-dependent formaldehyde dehydrogenase (FIG 3E) (Lidstrom, 2006). This dehydrogenase catalyzes the NAD^+-dependent oxidation of a spontaneously formed product of mycothiol and formaldehyde, *S*-hydroxymethyl-mycothiol, to *S*-formyl-mycothiol, which can subsequently be hydrolyzed to formate and mycothiol (Jothivasan & Hamilton, 2008; Newton *et al.*, 2008; Rawat & Av-Gay, 2007). Three possible pathways for conversion of *S*-formyl-mycothiol are known: spontaneous hydrolysis to formate and mycothiol, hydrolysis to formate and mycothiol by an *S*-formyl-mycothiol hydrolase (FMH), or oxidation by a molybdoprotein aldehyde dehydrogenase to *S*-carboxy-mycothiol, which spontaneously decomposes to carbon dioxide and mycothiol (Duine, 1999; Vogt *et al.*, 2003).

In methylotrophic organisms, formate dehydrogenases (FDH) catalyze the terminal step of the linear dissimilatory pathway to generate reducing equivalents. Methylotrophs usually contain multiple FDHs as reported for *M. extorquens*, which possesses four different FDH enzymes. This includes one NAD^+-dependent FDH containing at least one iron-sulfur cluster and a tungsten-cofactor (FDH1) (Laukel *et al.*, 2003), one predicted NAD^+-dependent FDH containing molybdenum (FDH2) (Chistoserdova *et al.*, 2004), one predicted periplasmic cytochrome-linked FDH (FDH3) (Chistoserdova *et al.*, 2004), and a novel type of FDH with an unknown electron acceptor which also contains a putative molybdenum-cofactor (FDH4) (Chistoserdova *et al.*, 2007). Only the latter one is essential for oxidation of formate to CO_2 during growth on methanol. The FDH1, FDH2 and FDH3 enzymes are needed during growth on formate as sole carbon source (Chistoserdova *et al.*, 2004; Chistoserdova *et al.*, 2007). However, FDHs are not only attributed to the methylotrophic metabolism, but can fulfill different physiological functions, e.g. in anaerobic respiratory pathways, in the fermentative metabolism or during chemoautotrophic growth, where they oxidize formate to CO_2 by transferring electrons to various acceptors such as NAD^+, $NADP^+$, cytochromes, cofactor F_{420}, fumarate or unknown electron acceptors (Chistoserdova *et al.*, 2007; Friedebold & Bowien, 1993; Kröger *et al.*, 1979; Schauer & Ferry, 1986; Yagi, 1979; Yamamoto *et al.*, 1983).

2.1.3 Assimilation of formaldehyde, CO_2 and/ or methylene-H_4F

In methylotrophic metabolism, the assimilation of formaldehyde, CO_2 and/or methylene-H_4F is essential for growth on C_1 carbon sources (Chistoserdova, 2011). In β- and γ-proteobacteria as well as in Gram-positive bacteria, assimilation of formaldehyde occurs via the ribulose monophosphate (RuMP) pathway (Brautaset *et al.*, 2004; Lidstrom, 2006). In methylotrophic yeasts, formaldehyde is assimilated via the xylulose monophosphate (XuMP) pathway (Kato *et al.*, 1982). Assimilation via the serine cycle occurs at the level of methylene-H_4F and CO_2 (Smejkalova *et al.*, 2010) and is restricted to α-proteobacteria (Lidstrom, 2006). An overview of these pathways is given in FIGs 4-6. In the group of α-proteobacteria, autotrophic methylotrophs can be found as well. These assimilate CO_2, which is generated during oxidation of methylated substrates, via the classical Calvin-Benson-Bassham (CBB) cycle (Baker *et al.*, 1998; Lidstrom, 2006) that is not further discussed here.

The RuMP pathway employs two specific key enzymes, the 3-hexulose-6-phosphate synthase (HPS) and the 6-phospho-3-hexuloisomerase (PHI), which catalyze the condensation of formaldehyde with ribulose-5-phosphate (Ru5P) to generate hexulose-6-phosphate (H6P) and its isomerization to fructose-6-phospate (F6P), respectively (Chistoserdova, 2011). Four variants of the RuMP pathway, differing in the mode of metabolization of the synthesized F6P and regeneration of the formaldehyde acceptor Ru5P, are known in methylotrophs. F6P is either converted to pyruvate and glyceraldehyde-3-phosphate (GAP) via enzymes of the Entner-Doudoroff pathway or it is converted to GAP and dihydroxyacetone phosphate (DHAP) via enzymes of glycolysis. During the regeneration of the C_1 acceptor Ru5P either a fructose bisphosphate aldolase/ seduheptulose bisphosphatase variant or a fructose bisphosphate aldolase/ transaldolase variant can be employed (Brautaset *et al.*, 2004; Heggeset *et al.*, 2012; Jakobsen *et al.*, 2006; Lidstrom, 2006). In non-methylotrophic organisms orthologous genes coding for HPS and PHI were found. These are induced in presence of formaldehyde and contribute to the detoxification of formaldehyde as shown for *B. subtilis* (Yasueda *et al.*, 1999). The RuMP pathway is a proven candidate to confer the capability of formaldehyde detoxification and assimilation to organisms that are used in biotechnological processes. It is more efficient than the other C_1 assimilation pathways and the enzymes participating in the RuMP pathway require no cofactors. E.g., *hps* and *phi* genes from methylotrophic organisms

were successfully transferred to *Pseudomonas putida* and *Burkholderia cepacia* to increase the formaldehyde tolerance and to establish the ability to assimilate formaldehyde, respectively (Koopman *et al.*, 2009; Mitsui *et al.*, 2003; Yurimoto *et al.*, 2009).

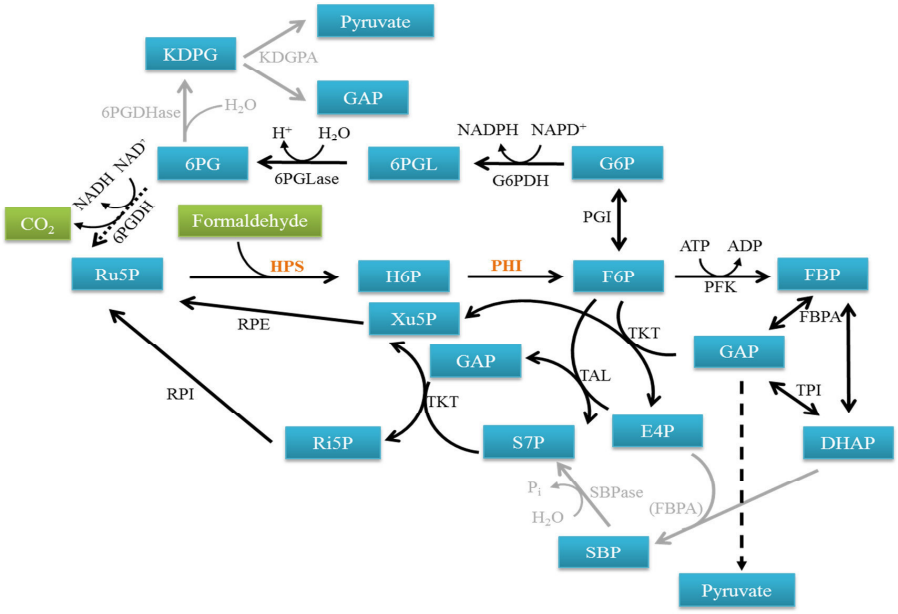

FIG 4 Overview of the different variants of the ribulose monophosphate (RuMP) pathway in methylotrophic bacteria. Key enzymes are the 3-hexulose-6-phosphate synthase (HPS) and the 6-phospho-3-hexuloisomerase (PHI) (shown in orange) which catalyze the condensation of formaldehyde with ribulose-5-phosphate (Ru5P) to form hexulose-6-phosphate (H6P) and its subsequent isomerization to fructose-6-phosphate (F6P), respectively. F6P is a central intermediate of this pathway. For energy generation it can be catabolized via glycolysis (Enzymes: PFK, phosphofructokinase; FBPA, fructose-1,6-bisphosphate aldolase; TPI, triosephosphate isomerase; Metabolites: FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate) or it can be converted in three steps to 6-phosphogluconate (Enzymes: PGI, phosphoglucoisomerase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGLase, 6-phosphoglucolactonase; Metabolites: G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone) which is converted via Entner-Doudoroff specific enzymes (shown in grey) to pyruvate and GAP (Enzymes: 6PGDHase, 6-phosphogluconate dehydratase; KDPGA, 2-keto-3-deoxy-6-phosphogluconate aldolase; Metabolites: KDPG, 2-keto-3-deoxy-6-phosphogluconate; pyruvate; GAP). For regeneration of the formaldehyde acceptor Ru5P and generation of reducing equivalents, F6P can be channeled into the cyclic RuMP pathway, which also includes the conversion of F6P to 6PG, but 6PG is subsequently oxidized to Ru5P via the 6-phosphogluconate dehydrogenase (6PGDH) (dotted line). Next to this pathway, regeneration of Ru5P is also performed via conversion of sugar phosphates catalyzed by transaldolase and transketolase (non-oxidative pentose-phosphate pathway). Two variants for this pathway exist: The FBPA/transaldolase variant and the FBPA/seduheptulose bisphosphatase variant shown in grey (Enzymes: TKT, transketolase; TA, transaldolase, RPE/RPI; ribose-5-phosphate epimerase/isomerase; SBPase, seduheptulose bisphosphatase; FBPA; Metabolites: GAP; F6P; E4P, erythrose-4-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; Ri5P, ribose-5-phosphate; DHAP; SBP, seduheptulose bisphosphate). (Heggeset *et al.*, 2012)

The XuMP pathway is found in methylotrophic yeasts, like *Candida biodinii* or *P. pastoris*, in which the dihydroxyacetone synthase (DAS, shown in orange) catalyzes the transfer of the glyceraldehyde group from xylulose-5-phosphate (Xu5P) to formaldehyde, generating dihydroxyacetone (DHA) and GAP. The DAS is located in the peroxisomal matrix assuming that the formaldehyde fixation takes place in the peroxisomes (Kato *et al.*, 1982; Yurimoto *et al.*, 2005). Following, DHA is phosphorylated via the dihydroxyacetone kinase (DAK) to DHAP. GAP and DHAP can be directly metabolized via glycolysis, but regeneration of Xu5P requires the conversion of DHAP and GAP to fructose-1,6-bisphosphate, which is dephosphorylated by a fructose bisphosphatase (FBPase) to F6P. Subsequently, F6P can enter the regeneration part of the XuMP pathway and can also be metabolized via glycolysis (Yurimoto *et al.*, 2005).

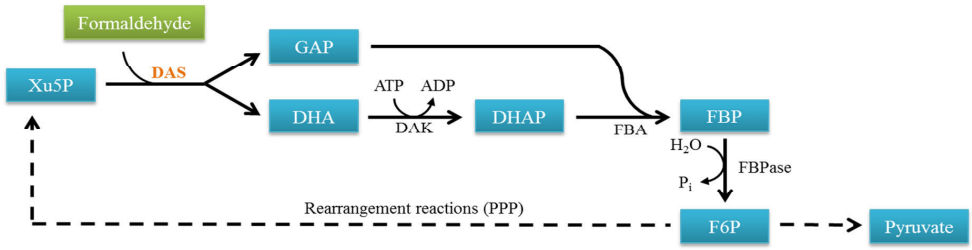

FIG 5 Overview of formaldehyde assimilation in methylotrophic yeasts via the xylulose monophosphate pathway (XuMP). The key step is the conversion of xylulose-5-phosphate (Xu5P) and formaldehyde to glyeraldehyde-3-phosphate (GAP) and dihydroxyacetone (DHA) via dihydroxyacetone synthase (DAS, shown in organge). DHA is subsequently phosphorylated via dihydroxyacetone kinase (DAK) to DHAP. For regeneration of Xu5P, DHAP and GAP are converted to fructose-1,6-bisphosphate (FBP) and subsequently to fructose-6-phosphate (F6P) via the fructose-1,6-bisphosphate aldolase (FBA) and the fructose-1,6-bisphosphatase (FBPase). The following rearrangement reactions catalyzed by enzymes of the non-oxidative pentose phosphate pathway (PPP) occur as already described for the RuMP pathway in FIG 3. Adapted from Yurimoto *et al.*, (2005).

In methylotrophic α-proteobacteria, the assimilation of C_1 compounds starts with the spontaneous condensation of formaldehyde and H_4F to form methylene-H_4F, which is subsequently introduced into the serine cycle via condensation with glycine (Fuchs, 2006) (FIG 6A). Recent publications revealed that in *M. extorquens* formaldehyde is not condensed with H_4F, but is oxidized via the H_4MPT-linked pathway to formate, which is either oxidized to CO_2 yielding additional reducing equivalents or is enzymatically condensed with H_4F to Formyl-H_4F. Formyl-H_4F is further reduced via two enzymatic steps to methylene-H_4F which is finally introduced into the serine cycle (Smejkalova *et al.*, 2010) (FIG 6B). Serine is converted via two

enzymatically steps to phosphoenolpyruvate (PEP) and via carboxylation and subsequent reduction followed by a ligase reaction, malyl-CoA is generated, which is cleaved into acetyl-CoA and glyoxylate. The latter metabolite is directly converted to the methylene-H4F acceptor glycine. Due to the deduction of biosynthetic precursors from the serine cycle a further glyoxylate regeneration pathway is necessary. Thus, acetyl-CoA is converted to glyoxylate either via the classic glyoxylate shunt or via the ethylmalonyl-CoA pathway (EMCP) (Erb *et al.*, 2007; Erb *et al.*, 2009; Smejkalova *et al.*, 2010). The serine cycle was intensively studied in *M. extorquens* and a comprehensive overview of this cycle including the EMCP is given in Anthony (2011) and Chistoserdova *et al.*, (2003).

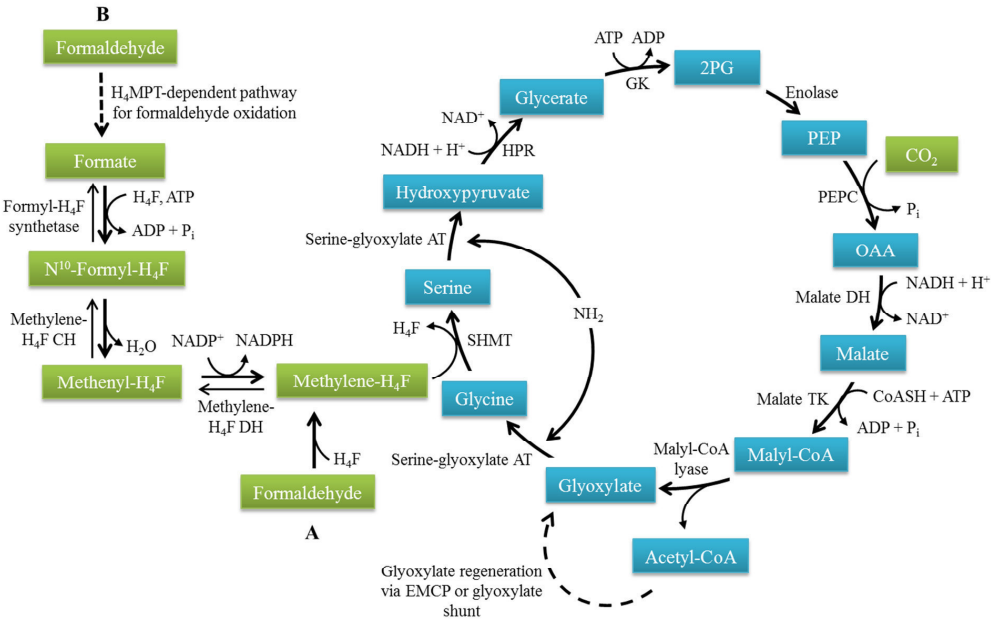

FIG 6 Serine pathway for assimilation of C1 compounds in methylotrophic bacteria. Methylene-H4F can be either directly formed via spontaneous condensation of formaldehyde with H4F (**A**) or formaldehyde is initially oxidized to formate via the H4MPT-dependent pathway and formate is afterwards reduced via three enzymatic reactions to methylene-H4F (**B**). Glycine serves as acceptor for methylene-H4F and regeneration of glyoxylate occurs either directly via conversion of malyl-CoA to glyoxylate and acetyl-CoA catalyzed by the malyl-CoA lyase or via the EMCP (ethylmalonyl-CoA pathway) or via the classical glyoxylate shunt. Abbreviations: 2PG, 2-phosphoglycerate; AT, aminotransferase; CH, cyclohydrolase; DH, dehydrogenase; GK, glycerate kinase; H4F, tetrahydrofolate; H4MPT, tetrahydromethanopterin; HPR, hydroxypyruvate reductase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; SHMT, serine hydroxymethyl transferase; TK, thiokinase. Adapted from (Smejkalova *et al.*, 2010).

2.2 *Corynebacterium glutamicum* as platform organism for methanol-based fermentation processes

C. glutamicum is a well-established organism in industrial biotechnology. Highly productive strains have been developed to produce several million tons of amino acids annually, in particular the feed additive L-lysine and the flavor enhancer L-glutamate. In addition, *C. glutamicum* is an efficient host for the production of heterologous proteins (Scheele *et al.*, 2013) and has been engineered for the production of a variety of other commercially interesting compounds (Becker & Wittmann, 2012; Zahoor *et al.*, 2012), such as organic acids (Litsanov *et al.*, 2012; Okino *et al.*, 2008; Wieschalka *et al.*, 2013), diamines (Kind & Wittmann, 2011; Mimitsuka *et al.*, 2007; Schneider & Wendisch, 2011), or alcohols (Blombach *et al.*, 2011; Inui *et al.*, 2004; Smith *et al.*, 2010). However, production titers and yields have to be further optimized for economic reasons. *C. glutamicum* is a Gram-positive, biotin-auxotrophic, predominantly aerobic and fast growing soil bacterium, which belongs to the mycolic acid-containing *Corynebacteriales* and is assigned to the family *Corynebacteriaceae* (Gao & Gupta, 2012). In terms of the number of genomes that have been sequences, the *Corynebacteriales* represent one of the largest groups within the actinobacteria and also includes human pathogens like *C. diphtheriae* and *Mycobacterium tuberculosis*. Hence, in case of cell wall biosynthesis, the well-studied, non-pathogenic bacterium *C. glutamicum* also serves as model organism for these pathogenic members of the *Corynebacteriaceae* and *Mycobacteriaceae* (Gao & Gupta, 2012). Originally it was isolated by Shigezo Udaka due to its ability to excrete glutamate under biotin-limiting growth conditions (Abe *et al.*, 1967; Kinoshita *et al.*, 1958; Udaka, 1960).

For industrial production processes with *C. glutamicum*, mainly sugars derived from starch (glucose) or from molasses (sucrose and fructose) are used as carbon sources (Kelle *et al.*, 2005; Kimura, 2005; Zahoor *et al.*, 2012). The natural substrate spectrum of *C. glutamicum* further includes sugars such as ribose, mannose and maltose, alcohols such as ethanol and inositol as well as organic acids such as pyruvate, acetate, L-lactate, propionate and gluconate (Zahoor *et al.*, 2012). Over the past years, *C. glutamicum* was genetically engineered towards the ability to efficiently utilize several cheaply available carbon sources, e.g. the TCA cycle intermediates malate, fumarate and succinate, the lignocellulose compounds arabinose and xylose as well as starch, cellobiose, glycerol, lactose, galactose and glucosamine [(Uhde *et al.*, 2013; Zahoor *et al.*,

2012) and references within]. While glucose is usually co-metabolized with other carbohydrates and organic acids, diauxic growth and sequential utilization of carbon sources was described for the mixture of glucose and glutamate as well as for glucose and ethanol (Arndt *et al.*, 2008; Kronemeyer *et al.*, 1995). The ethanol catabolism is subject to carbon catabolite control, involving the transcriptional regulators RamA and RamB.

Uptake and phosphorylation of the sugars glucose, sucrose and fructose occurs via the phosphotransferase system (Parche *et al.*, 2001) or, in the case of glucose, alternatively by *myo*-inositol permeases with subsequent phosphorylation by glucokinases (Lindner *et al.*, 2010; Lindner *et al.*, 2011). Further metabolism of the sugar phosphates occurs via glycolysis and the pentose phosphate pathway (PPP) before entering the citric acid cycle. The general role of the PPP is the supply of reducing power and precursors (e.g. NADPH, ribose-5-phosphate and erythrose-4-phosphate) for the biosynthesis of aromatic amino acids, such as tyrosine or phenylalanine or nucleotides (Yokota & Lindley, 2005). Ethanol is metabolized via an alcohol dehydrogenase and acetaldehyde dehydrogenase, which catalyze the two-step NAD^+-dependent oxidation to acetate. Subsequently, acetate is activated by an acetate kinase and a phosphotransacetylase to acetyl-CoA before it enters the citric acid cycle (Arndt *et al.*, 2008; Gerstmeir *et al.*, 2003; Kotrbova-Kozak *et al.*, 2007). The citric acid cycle provides precursor metabolites for biosynthetic processes and reducing equivalents to the respiratory system, where they serve as electron donors for oxidative phosphorylation. During growth on ethanol, the glyoxylate shunt plays an important role in anaplerosis. During growth on carbohydrates anaplerosis is accomplished by carboxylation of phosphoenolpyruvate and pyruvate to yield oxaloacetate (Bott, 2007; Eikmanns, 2005).

2.3 Aims of this dissertation

The relevance of methanol as feedstock in biotechnological processes is expected to increase in the coming years and development of low-cost production processes based on renewable carbon sources paves the way for the methanol-based bioeconomy (Olah, 2013; Schrader *et al.*, 2009). Therefore, the primary aim of this dissertation was the expansion of the substrate spectrum of the industrially important amino acid-producer *Corynebacterium glutamicum* towards the utilization of methanol. This included the selection and establishment of suitable enzymes catalyzing the oxidation of methanol to formaldehyde as well as the establishment of a pathway for the assimilation of formaldehyde into the central metabolism. In order to verify the functionality of the heterologous pathway for methanol utilization, the *in vitro* activities of the implemented enzymes were assayed and the constructed recombinant *C. glutamicum* strains were characterized. Growth studies were performed to characterize these strains regarding their ability to oxidize methanol and regarding their growth behavior in the presence of methanol. With the aim to verify the incorporation of methanol-derived carbon into intracellular metabolites serving as biomass precursor, ^{13}C-methanol labeling experiments and intracellular metabolite analysis were performed. Since initial studies on the response of *C. glutamicum* towards methanol revealed its ability to oxidize this C_1 compound, identification and biochemical characterization of contributing enzymes was a further aim of this study. In order to get hints on the possible physiological function and the regulation of the endogenous methanol oxidation, studies on global gene expression changes in the presence of methanol as well as characterization of generated mutants being unable to oxidize methanol, formaldehyde or formate were performed.

3 Results

The major topic of this PhD thesis was the engineering of *Corynebacterium glutamicum* towards the utilization of methanol as auxiliary substrate during sugar-based growth, since this industrially important amino acid-producer is a non-methylotrophic organism and hence not able to use C_1 compounds as carbon source. The obtained results have been summarized in three publications; one of them was submitted recently. The two accepted publications describe the identification of an endogenous pathway for oxidation of methanol to CO_2 as well as the subsequent characterization of enzymes involved in these reactions. For the first time, it could be shown, that *C. glutamicum* has the ability to oxidize formate. The results are described in the publication "*Corynebacterium glutamicum* harbours a molybdenum cofactor-dependent formate dehydrogenase which alleviates growth inhibition in the presence of formate". In the course of these studies, the formate dehydrogenase FdhF, the accessory protein FdhD and the putative molybdopterin guanine dinucleotide biosynthesis protein Cg0617 were shown to be responsible for the oxidation of formate. Further studies revealed that the FdhF is a molybdenum-cofactor dependent enzyme, but the involved electron acceptor could not be identified.

Additional investigations revealed that the formate dehydrogenase contributes to the endogenous pathway for methanol oxidation in *C. glutamicum*, which is presented in the publication "C_1 Metabolism in *Corynebacterium glutamicum*: an endogenous pathway for oxidation of methanol to carbon dioxide". In this publication the identification of enzymes involved in endogenous methanol oxidation as well as the regulation of this pathway is described. The obtained results show, that the alcohol dehydrogenase AdhA is mainly responsible for methanol oxidation and that the oxidation of formaldehyde is catalyzed predominantly by the acetaldehyde dehydrogenase Ald and the mycothiol-dependent formaldehyde dehydrogenase AdhE. In addition, the methanol catabolism was shown to be subject to carbon catabolite repression by glucose.

Based on these findings, *C. glutamicum* was engineered towards the assimilation of methanol-derived carbon into the biomass. The results are described in the manuscript "Metabolic engineering of *Corynebacterium glutamicum* for the metabolization of methanol". Implementation of a heterologous methanol dehydrogenase from *Bacillus methanolicus* resulted in a three-fold

increased methanol oxidation rate compared to the *C. glutamicum* wild type and implementation of the key enzymes of the ribulose monophosphate pathway, 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase, from *Bacillus subtilis* resulted in assimilation of the generated formaldehyde. ^{13}C-methanol labeling experiments revealed 3-10% m+1 labeling of selected intracellular metabolites in the *C. glutamicum* wild type background and in comparison to the growth on glucose alone, cultivation in glucose/methanol medium led to an slightly increased CDW (8.0 ± 0.05 mg/ml vs. 8.4 ± 0.03). Establishment of the synthetic pathway for methanol oxidation and formaldehyde assimilation in *C. glutamicum* Δ*ald*Δ*adhE*, a mutant which was previously identified to be severely impaired in its ability to oxidize formaldehyde, increased the m+1 labeling of intracellular metabolites to 8-25%.

In addition, ^{13}C-methanol labeling experiments revealed an unexpected high labeling fraction in the m+1 mass isotopomers of the amino acid L-glutamate, which was not included in the latter publication. In the recombinant *C. glutamicum* wild type strain expressing the genes for methanol oxidation and formaldehyde assimilation, labeling fractions of 3-10% in the m+1 mass isotopomers of various intracellular metabolites were detected, but up to 22% m+1 labeled L-glutamate was found. This situation was similar in the recombinant *C. glutamicum* Δ*ald*Δ*adhE* strain expressing same genes. Labeling fractions of 8-25% in the m+1 mass isotopomers of intracellular metabolites were detected, but L-glutamate was found to be 33% m+1 labeled.

3.1

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Corynebacterium glutamicum harbours a molybdenum cofactor-dependent formate dehydrogenase which alleviates growth inhibition in the presence of formate

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Here, we show that *Corynebacterium glutamicum* ATCC 13032 co-metabolizes formate when it is grown with glucose as the carbon and energy source. CO_2 measurements during bioreactor cultivation and use of ^{13}C-labelled formate demonstrated that formate is almost completely oxidized to CO_2. The deletion of *fdhF* (cg0618), annotated as formate dehydrogenase (FDH) and located in a cluster of genes conserved in the family *Corynebacteriaceae*, prevented formate utilization. Similarly, deletion of *fdhD* (cg0616) resulted in the inability to metabolize formate and deletion of cg0617 markedly reduced formate utilization. These results illustrated that all three gene products are required for FDH activity. Growth studies with molybdate and tungstate indicated that the FDH from *C. glutamicum* ATCC 13032 is a molybdenum-dependent enzyme. The presence of 100 mM formate caused a 25 % lowered growth rate during cultivation of *C. glutamicum* ATCC 13032 wild-type in glucose minimal medium. This inhibitory effect was increased in the strains lacking FDH activity. Our data demonstrate that *C. glutamicum* ATCC 13032 possesses an FDH with a currently unknown electron acceptor. The presence of the FDH might help the soil bacterium *C. glutamicum* ATCC 13032 to alleviate growth retardation caused by formate, which is ubiquitously present in the environment.

INTRODUCTION

Formate plays an important role in microbial metabolism. On the one hand, many micro-organisms such as methanogens and sulfate-reducing bacteria are able to use formate as a substrate for growth (Ferry, 2011; Plugge *et al.*, 2011). On the other hand, formate is an end product of bacterial fermentations (Ferry, 2011; Lin & Iuchi, 1991). Formate can be formed by the coenzyme A-dependent cleavage of pyruvate to yield formate and acetyl-CoA (Lin & Iuchi, 1991), by formaldehyde oxidation or by hydrolysis of 10-formyltetrahydrofolate (Misset-Smits *et al.*, 1997; Nagy *et al.*, 1995). Formate is a crucial compound in many syntrophic associations, where it is an intermediate in the conversion of biological polymers to methane and carbon dioxide (Crable *et al.*, 2011; McInerney *et al.*, 2009; Stams & Plugge, 2009).

In accordance with the multiple physiological roles of formate in various bacteria and archaea different types of formate dehydrogenases (FDHs) have evolved, thereby FDHs comprise a heterogeneous group of enzymes found in both prokaryotes and eukaryotes, which catalyse the oxidation of formate to CO_2. FDHs can be found in prokaryotic micro-organisms such as aerobic methylotrophs and chemoautotrophs, anaerobic or facultative anaerobic bacteria, as well as methanogenic archaea (Friedebold & Bowien, 1993; Jormakka *et al.*, 2002; Karzanov *et al.*, 1991; Schauer *et al.*, 1986). Depending on the type of FDH, the electrons are transferred to an acceptor such as NAD^+, $NADP^+$, cytochrome, cofactor F_{420}, fumarate, nitrate, sulfate or still unknown acceptors (Chistoserdova *et al.*, 2007; Friedebold & Bowien, 1993; Kröger *et al.*, 1979; Schauer *et al.*, 1986; Yagi, 1979; Yamamoto *et al.*, 1983; Jormakka *et al.*, 2003; Kröger *et al.*, 1986; Moura *et al.*, 2004; Sebban *et al.*, 1995). NAD^+-dependent FDHs are widely used as efficient biocatalysts for NADH regeneration in industrial applications and by the finding that the FDH is capable of selectively cleaving formic acid esters to the respective alcohol, FDH has emerged as a superior deformylation catalyst compared with hydrolases (Fröhlich *et al.*, 2011; Tishkov & Popov, 2004).

The industrial workhorse *Corynebacterium glutamicum* is well known for its ability to produce amino acids (Eggeling & Bott, 2005; Kabus *et al.*, 2007; Takors *et al.*, 2007;

Abbreviation: FDH, formate dehydrogenase.

Two supplementary figures and a supplementary table are available with the online version of this paper.

Wendisch *et al.*, 2006a, b) and has also been engineered for production of amino-acid-derived products, alcohols and organic acids from glucose and other carbon sources (Blombach *et al.*, 2011; Buschke *et al.*, 2011; Gopinath *et al.*, 2011; Litsanov *et al.*, 2012a, b; Niimi *et al.*, 2011; Rittmann *et al.*, 2008; Sasaki *et al.*, 2009; Schneider *et al.*, 2011, 2012; Stäbler *et al.*, 2011). In initial experiments on the use of different carbon sources by this organism, we observed that *C. glutamicum* ATCC 13032 could consume formate. In both genome sequences of *C. glutamicum* ATCC 13032 (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003), an FDH gene has been annotated with homology to formate dehydrogenase from *Escherichia coli*.

E. coli harbours three FDH isoenzymes. FdnGHI (FDH-N) and FdoGHI (FDH-O) are respiratory enzymes which work under anaerobic conditions in the presence of nitrate as terminal electron acceptor and are anchored to the periplasmic side of the inner membrane (Jormakka *et al.*, 2002; Thomé *et al.*, 2012). In the absence of an exogenous electron acceptor under anaerobic conditions, FdhF (FDH-H) is expressed as part of the formate–hydrogen lyase complex and is located at the cytoplasmic side of the membrane (Axley *et al.*, 1990; Boyington *et al.*, 1997; Raaijmakers & Romão, 2006). It delivers electrons from formate to hydrogenase 3 and protons are reduced to hydrogen molecules (Ingledew & Poole, 1984). The expression of the FDH-H is induced by formate and repressed by oxygen, nitrate, nitrite and other electron acceptors (Axley *et al.*, 1990; Pecher *et al.*, 1983). X-ray structure analysis of FdhF revealed the presence of a Fe_4S_4 cluster and a molybdenum cofactor in the catalytic subunit (Boyington *et al.*, 1997; Jormakka *et al.*, 2002). Furthermore, it is a selenoprotein in which selenium is covalently bound as a selenocysteine and the UGA codon directs the co-translational insertion into the polypeptide (Zinoni *et al.*, 1987).

The predicted formate dehydrogenase from *C. glutamicum* ATCC 13032 has not been shown, to our knowledge, to be involved in formate consumption. In this study we show that *C. glutamicum* ATCC 13032 is able to oxidize formate, identify genes involved in this reaction, and propose a physiological function.

METHODS

Bacterial strains, plasmids, media and growth conditions. Strains constructed or used in this study are listed in Table 1. *E. coli* DH5α (Life Technologies) was used for cloning purposes. Plasmid pK19mobsacB was used for construction of the defined in-frame deletion mutants (Schäfer *et al.*, 1994) *C. glutamicum* ATCC 13032 Δcg0618, *C. glutamicum* ATCC 13032 Δcg0616 and *C. glutamicum* ATCC 13032 Δcg0617. For complementation studies, strains *C. glutamicum* ATCC 13032 Δcg0618/pAN6-cg0618, *C. glutamicum* ATCC 13032 Δcg0616/pAN6-cg0616, *C. glutamicum* ATCC 13032 Δcg0617/pAN6-cg0617 and the reference strain *C. glutamicum* ATCC 13032 pAN6 were constructed. Plasmid pEKEx2-cg0618-strep contains the cg0618 coding region (including a StrepTag-II coding sequence at the 3′-end) plus a 400 bp upstream region carrying the native promoter of cg0618. This plasmid was transferred into *C. glutamicum* ATCC 13032 Δcg0618 and used for production and purification of a C-terminally Strep-tagged FdhF protein, which served to determine the amino terminus of FdhF.

Several media were used for cultivation of *C. glutamicum* ATCC 13032 (at 30 °C) and *E. coli* (at 37 °C) under oxic conditions. LB medium contained (l^{-1}) 10 g NaCl (Sigma-Aldrich), 10 g tryptone (BD) and 5 g yeast extract (BD). For selection against *sacB*, LB medium was supplemented with 10 % (w/v) sucrose (Sigma-Aldrich). BHI medium contained 37 g brain heart infusion l^{-1} (BD). BHIS medium represents BHI medium supplemented with 91 g sorbitol l^{-1} (AppliChem). For agar plates, 1.8 % (w/v) agar (BD) was added. CGXII medium for growth of *C. glutamicum* ATCC 13032 strains was prepared as described by Keilhauer *et al.* (1993). If necessary, the media contained kanamycin (25 μg ml^{-1} for *C. glutamicum* ATCC 13032, 50 μg ml^{-1} for *E. coli*). Formate was added to the culture medium as a 5 M potassium formate stock solution that was neutralized with HCl. Growth was determined by measuring OD_{600}.

Recombinant DNA work. The enzymes for recombinant DNA work were obtained from Roche Diagnostics or Fermentas. Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared as described by Eikmanns *et al.* (1994). Plasmids were isolated by using the QIAprep spin miniprep kit (Qiagen). *E. coli* was transformed by using the RbCl method (Hanahan, 1983), *C. glutamicum* ATCC 13032 by electroporation (van der Rest *et al.*, 1999). Routine methods such as PCR,

Table 1. Strains used or constructed in this study

Strain	Purpose	Reference or source
E. coli DH5α	Cloning	Life Technologies
C. glutamicum ATCC 13032	Wild-type reference	Abe *et al.* (1967)
C. glutamicum Δcg0618	In-frame deletion of *fdhF*	This study
C. glutamicum Δcg0618/pAN6-cg0618	Plasmid-based complementation of *fdhF*	This study
C. glutamicum Δcg0616	In-frame deletion of *fdhD*	This study
C. glutamicum Δcg0616/pAN6-cg0616	Plasmid-based complementation of *fdhD*	This study
C. glutamicum Δcg0617	In-frame deletion of cg0617	This study
C. glutamicum Δcg0617/pAN6-cg0617	Plasmid-based complementation of cg0617	This study
C. glutamicum pAN6	Vector control	Frunzke *et al.* (2008)
C. glutamicum Δcg0618/pEKEx2-cg0618-strep	Plasmid-based expression of FdhF for determination of the N-terminus	This study

restriction or ligation were carried out according to standard protocols (Sambrook *et al.*, 1989).

The oligonucleotides used for cloning were obtained from Operon and are listed in Table 2. The in-frame deletion mutants of *C. glutamicum* ATCC 13032 were constructed via a two-step homologous recombination procedure, as described previously (Niebisch & Bott, 2001). According to this procedure, for deletion of *cg0618*, the primers PΔcg0618_1, PΔcg0618_2, PΔcg0618_3 and PΔcg0618_4 were used. For deletion of *cg0616*, the primers PΔcg0616_1, PΔcg0616_2, PΔcg0616_3 and PΔcg0616_4 were used. For deletion of *cg0617*, the primers PΔcg0617_1, PΔcg0617_2, PΔcg0617_3 and PΔcg0617_4 were used. The resulting deletion mutants were verified by DNA sequencing using oligonucleotides binding outside the homologous regions used for deletion with pK19mobsacB (P_fdhF_left and P_fdhF_right for verification of *C. glutamicum* ATCC 13032 Δ*fdhF*; P_fdhD_left and P_fdhD_right for verification of *C. glutamicum* ATCC 13032 Δ*fdhD*; P_cg0617_left and P_cg0617_right for verification of *C. glutamicum* ATCC 13032 Δcg0617; Table 2).

For complementation studies, plasmid pAN6-cg0618 was constructed using primers Pcg0618_fw and Pcg0618_rv (plasmid provided by Boris Litsanov, from our institute). Plasmid pAN6-cg0616 was constructed using primers Pcg0616_fw and Pcg0616_rev. Plasmid pAN6-cg0617 was constructed using primers Pcg0617_fw and Pcg0617_rev. For expression of C-terminally Strep-tagged protein Cg0618, the plasmid pEKEx2-cg0618-strep was constructed using primers Pcg0618_strep_fw and Pcg0618_strep_rev. The constructed plasmids were verified by DNA sequencing using plasmid-specific primers.

Determination of glucose and formate concentration. To determine glucose and formate concentration, HPLC analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies) with a cation exchange column (Organic acid Refill-column, 300 × 8 mm, CS-Chromatographie Service). Supernatants of *C. glutamicum* ATCC 13032 cultures were obtained by centrifugation of cell suspensions in 1.5 ml reaction tubes. If necessary, cell-free samples were diluted to the linear range of detection. The substances were eluted within 38 min with 100 mM sulfuric acid at a constant flow rate of 0.4 ml min^{-1} at 40 °C. The eluted organic acids were detected by a diode array detector (DAD G1315B) at a wavelength between 190 and 400 nm. Glucose was detected by a refractive index detector (RID G1362A). Concentrations were calculated from peak areas using calibration with external standards. Uptake rates were calculated in nmol min^{-1} (mg dry weight)$^{-1}$ (Frunzke *et al.*, 2008).

Determination of $^{13}CO_2$ and $^{12}CO_2$. To check and monitor the conversion of formate to CO_2 by *C. glutamicum* ATCC 13032 strains in ^{13}C labelling experiments, cells were grown in CGXII medium in a DASGIP Parallel Bioreactor System (DASGIP). During cultivation, pH, dissolved oxygen concentration and off-gas (CO_2 and O_2) were monitored online via the DASGIP Monitoring System (DASGIP Control 4.0). The pH of 7.0 was controlled by PID control loops and regulated by addition of 3 M KOH or 20 % (v/v) phosphoric acid via peristaltic pumps. O_2 saturation of >30 % in the medium was

Table 2. Primers used for cloning

Underlined nucleotides mark the restriction site.

Primer name	DNA sequence (5′–3′)	Restriction enzyme
PΔcg0618_1	GACCTGCAGAATTTGAAGATGCAAGC	*Pst*I
PΔcg0618_2	CCCATCCACTAAACTTAAACATGGTTTATTCATGGTGAGCAACGG	
PΔcg0618_3	TGTTTAAGTTTAG TGGATGGGCCAGTGTCCAAGTCAGTTGTG	
PΔcg0618_4	CAGGTCGACG AGCATTAACCAACGTGGAC	*Sal*I
P_fdhF_left	CTGTAATGTGCATTAGAGCGC	
P_fdhF_right	ACACAACGCGTGGGACCTG	
PΔcg0616_1	CAGTTGTGGTTCGCCTTGAAGCTTGAC	*Hin*dIII
PΔcg0616_2	CCCATCCACTAAACTTAAACACCGACCCATTTTTATTTAGCCTCC	
PΔcg0616_3	TGTTTAAGTTTAGTGGATGGGCTTGCTGGTTTTGTTCGGGGC	
PΔcg0616_4	CTGTAGATCAGCGAAAGTATGAGGGATCCGTC	*Bam*HI
P_fdhD_left	CTGAGGCCAACGTATTGGTTC	
P_fdhD_right	TGAAGGTTAGGCCTCGAGGAGG	
PΔcg0617_1	GACAAGCTTAACCAATGTGATTGAGCTACCC	*Hin*dIII
PΔcg0617_2	CCCATCCACTAAACTTAAACACGTTTCTGATGATGGCTGCAATTG	
PΔcg0617_3	TGTTTAAGTTTAGTGGATGGGAATGCTCTGCATCTTCAAGAAATC	
PΔcg0617_4	CAGGTCGACTCAACTG CTCAATAGACGTCG	*Sal*I
P_cg0617_left	GAGCGCATCTTCAACACATC	
P_cg0617_right	ATCCGAGGATCCGGTGTAATC	
Pcg0618_fw	TATACATATGACAACCCCTCCAACTGAG	*Nde*I
Pcg0618_rv	TATAGCTAGCCTAAGAAGCAGTACGTCCTGTTG	*Nhe*I
Pcg0616_fw	GACCATATGGGTCGG ATTACCCAAAAC	*Nde*I
Pcg0616_rev	GTCGCTAGCTTATCCGAGCTCGCC CGCATAG	*Nhe*I
Pcg0617_fw	GACCATATGTTGCAGCCATCATCAGAAACGAG	*Nde*I
Pcg0617_rev	GTCGCTAGCTTATTTAGCCTCCGGGTAATTTTTAG	*Nhe*I
Pcg0618_strep_fw	GACCCTGCAGGTTGAACCTTT CATTTTCAATTCTG	*Sbf*I
Pcg0618_strep_rev	GACGGTACCCTACTTCTCGAACTGTGGGTGGGACCAAGAAGCAGTACGTCCTGTTG	*Kpn*I

obtained by a cascaded dissolved oxygen control with agitation speeds of 400–1200 r.p.m. and a constant gas flow rate of 6 (standard litre) h^{-1}. If required, antifoam 204 (Sigma Life Science) was added. Growth was monitored offline by OD_{600} measurements. ^{13}C-Labelled sodium formate (Sigma-Aldrich) was added to the culture medium as stock solution. $^{13}CO_2$ and $^{12}CO_2$ off-gas analysis and quantification was performed with an FT-IR gas analyser (GASMET CR-2000i, Ansyco) between 900 and 4200 cm^{-1}, and the Calcmet software (Version 10).

Production, purification and MS analysis of C-terminally Strep-tagged FdhF. *C. glutamicum* ATCC 13032 Δcg0618 carrying plasmid pEKEx2-cg0618-strep was grown in 2000 ml baffled Erlenmeyer flasks filled with 300 ml CGXII medium and 25 μg kanamycin ml^{-1}. The culture was inoculated to OD_{600} 1 and induced with 0.7 mM IPTG (AppliChem) at OD_{600} 1.5. After 4 h of induction, the cells were harvested by centrifugation at 5000 ***g*** (30 min, 4 °C) and washed with TE buffer (100 mM Tris, 1 mM EDTA, pH 8.0). The cell pellet was resuspended in 8 ml TE buffer containing one protease inhibitor tablet (complete Mini-EDTA-free, Roche Diagnostics) and cells were disrupted via five passages through a French press (AMINCO Spectronic Instruments) at a pressure of 172 MPa. The protein-containing soluble fraction was separated from cell debris by a first centrifugation at 6000 ***g*** (20 min, 4 °C) and a second at 50 000 ***g*** (60 min, 4 °C). The supernatant was used for Strep-Tactin affinity chromatography and was incubated with 10 μl tetrameric avidin solution (5 μg μl^{-1}; Sigma-Aldrich) for 30 min on ice for saturation of biotinylated proteins. Afterwards, the protein solution was added to a 5 ml polypropylene column (Qiagen) containing 2 ml Strep-Tactin Sepharose (Strep-Tactin Superflow, Qiagen). After column equilibration with 4 ml buffer W (100 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0) the supernatant was applied onto the column for binding of the tagged protein to Strep-Tactin. The matrix was washed with 20 ml buffer W to remove non-specifically bound proteins. The elution of specifically bound proteins was performed with 8 × 1 ml buffer E (100 mM Tris, 1 mM EDTA, 400 mM NaCl, 2.5 mM desthiobiotin, pH 8.0). The elution fractions were separated by SDS-PAGE and visualized by Coomassie staining. The protein band at the expected size of tagged FdhF was subjected to in-gel digestion with trypsin. The peptides were eluted and subjected to MALDI-TOF-MS as described by Schultz *et al.* (2009).

RESULTS

Influence of formate on growth of *C. glutamicum* ATCC 13032 and consumption of formate

To test the influence and the fate of formate in cultures of *C. glutamicum* ATCC 13032 under oxic conditions, potassium formate was added, in concentrations up to 100 mM, to minimal medium containing 111 mM glucose (2 % w/v). The supplementation with formate resulted in a concentration-dependent decrease of the growth rate, whereas the final cell density was unaffected. The growth rate without formate was 0.43 h^{-1}, with 10 mM formate 0.41 h^{-1}, with 40 mM 0.37 h^{-1}, with 70 mM 0.36 h^{-1} and with 100 mM 0.32 h^{-1}. The concentration of formate leading to half-maximal growth inhibition of *C. glutamicum* ATCC 13032 wild-type was determined to be about 290 mM. When 100 mM KCl was added instead of potassium formate, the growth rate was 0.43 h^{-1}, showing that the inhibitory effect was caused by formate. Within 24 h of cultivation, formate was completely consumed by *C. glutamicum* ATCC 13032 (Fig. 1), as was glucose (data not shown). As shown below, formate consumption was dependent on the presence of the cells. The specific formate consumption rates were 28–30 nmol min^{-1} (mg dry weight)$^{-1}$, independent of the initial formate concentration. Interestingly, formate consumption was independent of growth, as it continued after the cells had reached the stationary phase. The observation that the final OD_{600} was not influenced by the presence of formate suggested that it is not assimilated in significant amounts.

FDH gene cluster and computational analysis of cg0618

The fact that *C. glutamicum* ATCC 13032 is able to metabolize formate gave rise to questions about the genes and proteins responsible for formate consumption. In the genome of *C. glutamicum* ATCC 13022 the ORF cg0618 has been annotated as a putative FDH and the gene was named *fdhF* (Kalinowski *et al.*, 2003). Immediately downstream of *fdhF*, two further genes related to FDH are located, cg0617 and cg0616 (*fdhD*), annotated as a putative molybdopterin guanine dinucleotide biosynthesis protein and an FDH accessory protein, respectively. A similar genetic organization is also found in other species of the suborder *Corynebacterineae*, e. g. in *Corynebacterium efficiens*, *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Rhodococcus erythropolis* (Fig. 2). In the genomes of the latter three species, however, there are no genes homologous to cg0617 based on sequence similarity, but genes homologous to molybdopterin guanine dinucleotide synthase MobA from *E. coli* can be found elsewhere in these genomes (Rv2453c, Mb2480c and RER_22150). It should be noted that there is no significant homology of

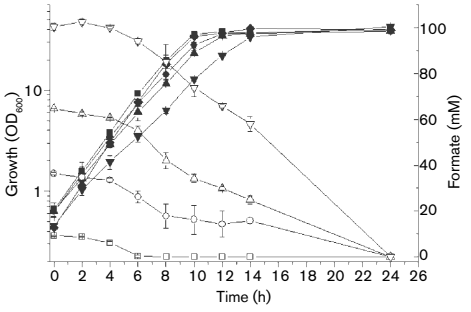

Fig. 1. Influence of formate on growth of *C. glutamicum* ATCC 13032 wild-type (OD_{600}, left axis, closed symbols) under oxic conditions in 60 ml CGXII medium containing 111 mM glucose and 0 mM (◆), 10 mM (■), 40 mM (●), 70 mM (▲) or 100 mM (▼) formate. Open symbols represent the formate concentration in the supernatant of the cultures (right axis). Mean ± SD values from two replicate experiments are shown.

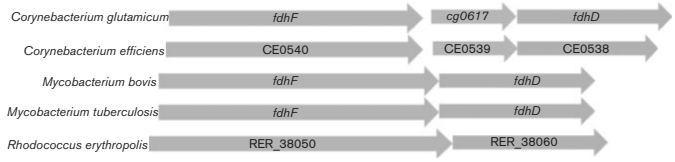

Fig. 2. Genetic organization of *fdhF* (cg0618) and *fdhD* (cg0616) in *C. glutamicum* ATCC 13032 and homologues in other species of the suborder *Corynebacterineae*. The genes *fdhF*, CE0540 and RER_38050 encode a putative formate dehydrogenase. The genes *fdhD*, CE0538 and RER_38060 encode a putative formate dehydrogenase accessory protein. The genes cg0617 and CE0539 encode a hypothetical protein annotated as a putative molybdopterin guanine dinucleotide biosynthesis protein.

the cg0617 protein from *C. glutamicum* ATCC 13032 to the MobA protein from *E. coli*, but cg1350 located elsewhere in the genome shows significant homology to MobA.

We used the amino acid sequence of the putative formate dehydrogenase encoded by *fdhF* in similarity searches using the NCBI BLAST tool. Relatively low sequence identities (22–29 %, BLAST tool, NCBI) were found to experimentally characterized FDHs participating in methylotrophic (Chistoserdova *et al.*, 2004, 2007), chemoautotrophic (Bowien & Kusian, 2002; Müller *et al.*, 1978), respiratory (Jormakka *et al.*, 2002) or fermentative (Raaijmakers & Romão, 2006) metabolism. Higher sequence identities were found with non-characterized FDHs from different *Streptomycetaceae* (e.g. 52 % with FDH from *Streptomyces coelicolor* and *S. lividans*) and from different mycobacteria including the human pathogen *M. tuberculosis* (48 %). However, a high sequence identity of 46 % also exists with FDH4 from *Methylobacterium extorquens*, which has been characterized as a molybdenum-dependent FDH with an important role in methanol metabolism (Chistoserdova *et al.*, 2007).

Using the NCBI conserved domain (CD) search tool (Marchler-Bauer *et al.*, 2011), the amino acid sequence of FdhF from *C. glutamicum* ATCC 13032 was shown to possess similarity to CDs of the FDH-alpha-like multidomain family and molybdopterin-binding superfamily, respectively. The molybdenum cofactor is required for functioning of many bacterial molybdoenzymes, including FDHs. Crystal structures of molybdoenzymes revealed amino acid residues involved in the binding of molybdopterin guanine dinucleotide (MGD) cofactor. In FDH-H from *E. coli* 35 residues coordinate the MGD cofactor, of which 23 are well-conserved in known MGD-containing FDHs (Boyington *et al.*, 1997). To identify conserved amino acids in FdhF from *C. glutamicum* ATCC 13032 that possibly bind the molybdenum cofactor, we compared the protein sequences of FdhF from *C. glutamicum* ATCC 13032, the well-characterized FDH-H from *E. coli*, and further FDH enzymes of the molybdopterin-binding superfamily. The protein sequence alignment shown in Fig. S1 (available with the online version of this paper) revealed at least 11 conserved or related amino acids in FdhF from *C. glutamicum* ATCC 13032 that could be involved in the coordinated binding of the molybdenum cofactor. Furthermore, the sequence alignment revealed five conserved cysteine residues in FdhF, four of which could be involved in the coordination of an Fe_4S_4 cluster (Cys-63, Cys-66, Cys-82, Cys-195; Fig. S1). The fifth conserved cysteine found in FdhF from *C. glutamicum* ATCC 13032 (Cys-199; Fig. S1) could be essential for catalytic activity, similar to a conserved cysteine or serine in other molybdenum cofactor-dependent enzymes or selenocysteine, as in the FDH-H from *E. coli* (Boyington *et al.*, 1997).

Deletion of genes in the FDH gene cluster and influence on formate consumption

To study the relevance of cg0618 (*fdhF*), cg0617 and cg0616 (*fdhD*) for formate consumption, the in-frame deletion mutants *C. glutamicum* ATCC 13032 Δcg0618, *C. glutamicum* ATCC 13032 Δcg0617 and *C. glutamicum* ATCC 13032 Δcg0616 were created as described in Methods. The deletions were confirmed by PCR analysis of the corresponding genomic regions and sequencing of the PCR products.

The deletion of cg0618 led to the inability of *C. glutamicum* ATCC 13032 to consume formate in defined medium with glucose (Fig. 3a). Moreover, during the course of the cultivation, *C. glutamicum* ATCC 13032 Δcg0618 showed slower growth compared with the wild-type, which could be attributed to the constantly high level of formate in the medium causing growth inhibition. In short-term (10 h) growth experiments we did not detect growth differences between *C. glutamicum* ATCC 13032 Δcg0618 and the wild-type in the absence of formate. To check whether there is a difference in growth in the long run, we performed long-term fitness experiments with two different initial mixtures of *C. glutamicum* ATCC 13032 wild-type and Δcg0618 mutant cells (40/60 % and 60/40 %) for direct comparison. The mixed cells were exponentially grown under oxic conditions for 6 days (144 h) by repeated inoculation into new shake flasks containing fresh

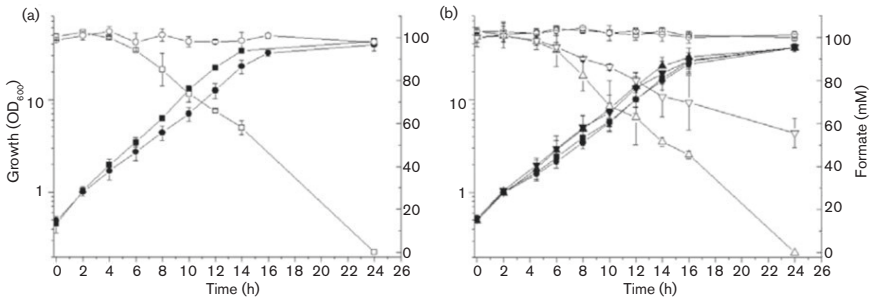

Fig. 3. Growth (left axes) and formate consumption (right axes) of different *C. glutamicum* ATCC 13032 strains under oxic conditions in minimal medium with 111 mM glucose and 100 mM formate. (a) OD_{600} (filled symbols) and formate concentration (open symbols) of the wild-type (■) and the deletion mutant Δcg0618 (●). (b) OD_{600} and formate concentration of strain Δcg0618/pAN6-cg0618 cultivated without (▲) or with (▼) 20 μM IPTG, and of the reference strain Δcg0618/pAN6 without (●) or with (■) IPTG. Mean ± SD values from two replicate experiments are shown.

medium with 111 mM glucose. Total genomic DNA from the initial (0 h) and the resulting (144 h) cell mixtures were analysed by quantitative real-time PCR to determine the relative content of the Δcg0618 locus compared with the *recF* locus which is present in both strains (data not shown). In both mixtures, we found a similar decrease in the relative Δcg0618 content after 144 h in both conditions, in the absence and in the presence of formate. The decrease corresponds to a growth rate of the Δcg0618 mutant which is about 3 % lower than the growth rate of the wild-type when grown in mixtures.

To exclude the possibility that the phenotype of *C. glutamicum* ATCC 13032 Δcg0618 was due to a secondary mutation, the mutant was transformed with the *fdhF* expression plasmid pAN6-cg0618 or, as a control, with pAN6. *C. glutamicum* ATCC 13032 Δcg0618/pAN6-cg0618, but not the control Δcg0618/pAN6, regained the ability to utilize formate (Fig. 3b). Again, the strain able to utilize formate showed faster growth over time than the strain unable to utilize it.

Strain Δcg0616 showed the same phenotype as Δcg0618, as it was completely unable to utilize formate (Fig. 4). The deficiency could be reversed by plasmid-borne expression of cg0616 using plasmid pAN6-cg0616, whereas plasmid pAN6 did not restore the ability to utilize formate. This shows that besides cg0618 cg0616 is also essential for formate utilization. Deletion of cg0617 did not cause a complete inability to utilize formate, but led to a decelerated formate consumption rate of about 6 nmol min^{-1} (mg dry weight)$^{-1}$, with a remaining formate concentration of about 60 mM after 29 h of cultivation (Fig. 4). Complementation of mutant Δcg0617 with plasmid pAN6-cg0617 restored the wild-type formate consumption rate of 30 nmol min^{-1} (mg dry weight)$^{-1}$. Again, the complemented strain showed better growth than the reference strain Δcg0617/pAN6.

Functional studies on the FDH from *C. glutamicum* ATCC 13032

To prove the conversion of formate to CO_2 by *C. glutamicum* ATCC 13032, ^{13}C-labelled sodium formate was used. *C. glutamicum* ATCC 13032 Δcg0618 and the wild-type were cultivated under controlled conditions in bioreactors using glucose minimal medium with and without formate. In a preliminary test, the $^{12}CO_2$ production rates of the two strains in the presence of ^{12}C-labelled formate were calculated from the monitored $^{12}CO_2$ concentrations in the off-gas. The wild-type showed a higher $^{12}CO_2$ production rate [137 nmol min^{-1} (mg dry weight)$^{-1}$] than the mutant strain Δcg0618 [105 nmol min^{-1} (mg dry weight)$^{-1}$], supporting the participation of FdhF in formate-dependent CO_2 production. In a second series of experiments, both strains were grown in glucose minimal medium supplemented with 70 mM sodium ^{13}C-labelled formate, and $^{13}CO_2$ production was monitored with an FT-IR gas analyser able to differentiate between $^{13}CO_2$ and $^{12}CO_2$. *C. glutamicum* ATCC 13032 Δcg0618 did not consume formate under controlled conditions, whereas formate was fully consumed by the wild-type within 12 h of cultivation (Fig. 5a). The calculated formate consumption rate was about 43 nmol min^{-1} (mg dry weight)$^{-1}$. The wild-type generated $^{13}CO_2$ whereas *C. glutamicum* ATCC 13032 Δcg0618 did not and the formation of $^{13}CO_2$ ceased with the depletion of ^{13}C-labelled formate after 12 h of cultivation (Fig. 5b). Overall, the calculated amount of $^{13}CO_2$ formed correlated with the total consumption of ^{13}C-labelled formate. In 12 h of cultivation, 70 mM ^{13}C-labelled formate was consumed and 67.3 mM $^{13}CO_2$ was produced. A gap in the $^{13}CO_2$ balance is expected since some $^{13}CO_2$ should be fixed in the biomass via the anaplerotic carboxylases. However, the result confirms that ^{13}C-labelled formate is directly oxidized to $^{13}CO_2$. The production of $^{12}CO_2$ was very similar in both cultures (Fig. 5b).

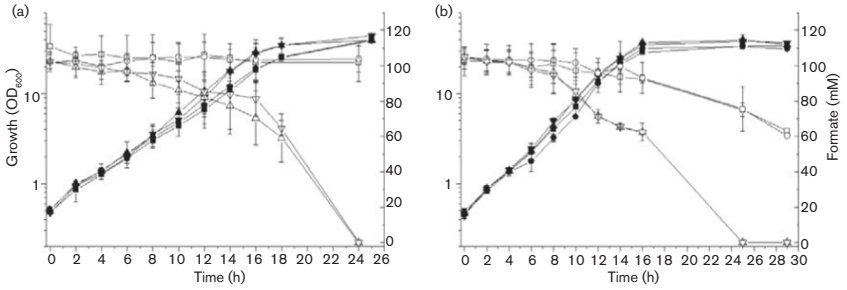

Fig. 4. Growth (left axes) and formate consumption (right axes) of different *C. glutamicum* ATCC 13032 strains under oxic conditions in minimal medium containing 111 mM glucose and 100 mM formate. (a) OD_{600} (filled symbols) and formate concentration (open symbols) of strain Δcg0616/pAN6-cg0616 without (▲) or with (▼) 20 μM IPTG and of the reference strain Δcg0616/pAN6 without (■) or with (●) 20 μM IPTG. (b) OD_{600} and formate concentration of strain Δcg0617/pAN6-cg0617 cultivated without (▲) or with (▼) 20 μM IPTG and of the reference strain Δcg0617/pAN6 without (■) or with (●) 20 μM IPTG. Mean ± SD values from two replicate experiments are shown.

Cofactor dependency and translational start of FdhF

The amino acid sequence analyses described above indicated that the *C. glutamicum* ATCC 13032 FdhF protein encoded by cg0618 contains a molybdopterin cofactor. To get further evidence for this assumption, the influence of sodium molybdate and sodium tungstate on formate consumption by the wild-type was tested (Fig. 6). It is known that the pterin can, in principle, bind either molybdenum or tungsten to form the biologically active molybdenum or tungsten cofactor and that the exchange of molybdenum and tungsten leads to inactive enzymes (McMaster & Enemark, 1998). As shown in Fig. 6, the presence of 2 μM sodium tungstate strongly inhibited formate consumption and this inhibition could be prevented by simultaneous addition of 2 μM sodium molybdate. Thus, the FDH from *C. glutamicum* ATCC 13032 appears to be a molybdenum-dependent enzyme.

In the two genome sequences published for *C. glutamicum* ATCC 13032, two different lengths were predicted for *fdhF*. The annotation by Ikeda & Nakagawa (2003) predicted a protein of 711 amino acids (cgl0529, NCgl0507), whereas the annotation by Kalinowski *et al.* (2003) predicted a protein of 762 amino acids (cg0618). To determine the

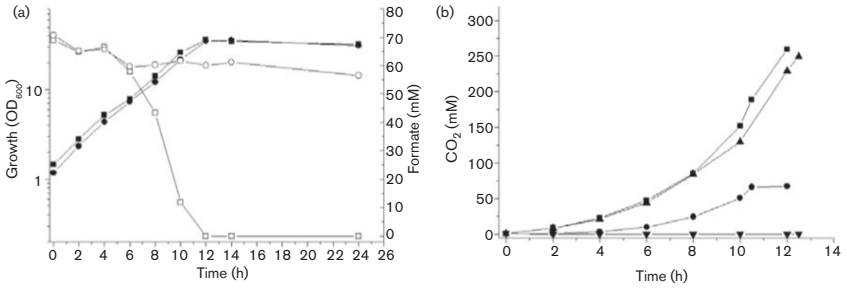

Fig. 5. Growth (left axis) and formate consumption (right axis) (a) and formation of $^{13}CO_2$ and $^{12}CO_2$ (b) of *C. glutamicum* ATCC 13032 wild-type and the deletion mutant Δcg0618 during cultivation under oxic conditions in DASGIP bioreactors containing minimal medium with 111 mM glucose and 70 mM ^{13}C-labelled sodium formate. (a) OD_{600} (closed symbols) and formate concentration (open symbols) for the wild-type (squares) and mutant Δcg0618 (circles). (b) The amount of $^{13}CO_2$ (●, ▼) and $^{12}CO_2$ (■, ▲) generated by *C. glutamicum* ATCC 13032 wild-type (●, ■) and mutant strain Δcg0618 (▼, ▲) as calculated from the off-gas analysis.

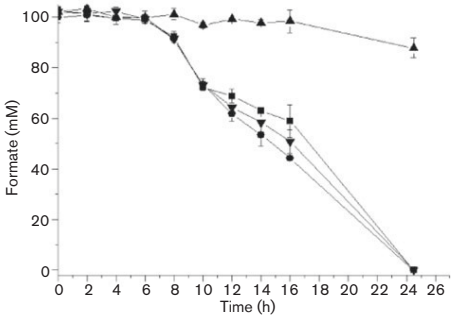

Fig. 6. Formate consumption of *C. glutamicum* ATCC 13032 wild-type under oxic conditions in minimal medium with 111 mM glucose and 100 mM formate without supplements (■), or supplemented with either 2 μM sodium tungstate (▲), 2 μM sodium molybdate (●) or 2 μM of each of the salts (▼). Mean ± SD values from two replicate experiments are shown.

amino terminus of native FdhF, C-terminally Strep-tagged FdhF was produced in *C. glutamicum* ATCC 13032 Δcg0618 using plasmid pEKEx2-cg0618-strep, which contains besides the *fdhF* coding region 400 bp upstream of the start codon of the 762 amino acid protein. After purification by StrepTactin affinity chromatography, the desthiobiotin eluate contained a dominant protein band of about 80 kDa as shown by SDS-PAGE and Coomassie staining (data not shown). The band was excised and after tryptic digestion was used for peptide mass fingerprint analysis by MALDI-TOF MS. The protein on hand was identified as FdhF by 31 matched mass peaks, corresponding to 43 % sequence coverage (Table S1 and Fig. S2). The mass with an *m*/*z* ratio of 2742.3 corresponded to the N-terminal peptide of the 762 amino acid FdhF protein with the N-terminal methionine cleaved off. The mass with an *m*/*z* ratio of 2108.2 corresponded to the peptide covering amino acids 27–47 of the 762 amino acid FdhF protein. These results show that native FdhF is a protein composed of 761 amino acids with the N-terminal sequence TTPPTEI.

DISCUSSION

From the central position of formate in the microbial world and its ubiquitous presence in the environment, it is plausible that soil bacteria harbour FDHs to be able to use formate as an energy source, and possibly for detoxification to alleviate growth retardation. Carboxylic acids such as formate, acetate and propionate can inhibit bacterial cell growth resulting in cellular responses including expression changes (Lee *et al.*, 2006; Litsanov *et al.*, 2012a; Polen *et al.*, 2003). *C. glutamicum* ATCC 13032 also showed reduced growth rates with increasing formate concentrations (Fig. 1). A possible explanation for the inhibitory effect is that formic acid (pK_a 3.77), but not its anion, can diffuse across the cytoplasmic membrane and thereby dissipate ion gradients and, dependent on the pH gradient, increase the internal anion concentration (Russell, 1992; Zaldivar & Ingram, 1999). However, with a pK_a of 3.77, formate exists predominantly in the deprotonated anionic form at physiological pH, necessitating a transport system to pass formate through the cell membrane. In *E. coli* the integral membrane protein FocA, which is impermeable to water but allows the passage of formate, was identified (Suppmann & Sawers, 1994; Wang *et al.*, 2009). In *C. glutamicum* ATCC 13032 no FocA-like sequence was found by BLAST analysis and the formate transport in *C. glutamicum* remains to be studied.

Our results show that an FDH is present in *C. glutamicum* ATCC 13032 and probably other related micro-organisms including *M. tuberculosis*. The FDH somewhat alleviated growth inhibition of *C. glutamicum* ATCC 13032 in the presence of formate, and *fdhF* and *fdhD* were essential for formate metabolism. No other formate-converting enzyme appeared to be present in *C. glutamicum* ATCC 13032 under the conditions tested. Also, BLAST analysis did not reveal other promising candidate formate dehydrogenase genes in the genome of *C. glutamicum* ATCC 13032.

The *fdhD* gene is annotated as an FDH accessory protein and is conserved in the *Corynebacterineae* and can also be found in other genomes. In *E. coli*, FdhD was found to be essential for activity of the two respiratory FDHs FDH-N and FDH-O as well as for the fermentative FDH-H (Abaibou *et al.*, 1995; Schlindwein *et al.*, 1990; Stewart *et al.*, 1991). Recently, FdhD from *E. coli* was shown to interact with IscS, one of the three *E. coli* L-cysteine desulfurases (Thomé *et al.*, 2012). The interaction of IscS with FdhD results in a sulfur transfer between IscS and FdhD in the form of persulfides. Substitution studies on the strictly conserved residues Cys-121 and Cys-124 of FdhD from *E. coli* showed that both are essential for the function of FdhD which was assessed through the FdhF activity (Thomé *et al.*, 2012). Corresponding cysteine residues are also present within the conserved motif Cys-Gly-Val-Cys-Gly in the FdhD proteins from *C. glutamicum* ATCC 13032 (Cys-151, Cys-154), *C. efficiens* YS-314, *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *Rhodococcus erythropolis* PR4 and others (data not shown). It is very likely that FdhD from *C. glutamicum* ATCC 13032 also acts as a sulfur transferase between a desulfurase and FdhF and therefore is essential for the formation of an active FDH. Several homologues of IscS from *E. coli* can also be found in the genomes mentioned above and have also been described in *C. glutamicum* ATCC 13032 (cg1214, cg1388 and cg1761) (see Schaffer *et al.*, 2001; Marienhagen *et al.*, 2005; Teramoto *et al.*, 2010).

Our experimental results and computational sequence analysis of FdhF indicate that it is a molybdenum-dependent

formate dehydrogenase which presumably contains an iron–sulfur cluster. Conserved cysteine residues were identified that are possibly involved in the coordination of an Fe_4S_4 cluster (Fig. S1). These residues are also conserved in FDH2 from *Methylobacterium extorquens* and FDH-S from *Ralstonia eutropha* (Fig. S1). The latter is known to contain Fe_4S_4 clusters (Chistoserdova *et al.*, 2004; Friedebold & Bowien, 1993). However, the presence of the iron–sulfur cluster in FdhF from *C. glutamicum* ATCC 13032 has still to be verified experimentally. The sequence analyses also revealed conserved residues for binding of the molybdopterin cofactor which can either bind molybdenum or tungsten to create the biologically active form. Tungsten-dependent enzymes were mainly found in anaerobic, thermophilic bacteria and archaea present in deep-sea hydrothermal vents, where tungsten is enriched. In most other habitats, tungsten is usually 100-fold less abundant than molybdenum (Hille, 2002; Schwarz *et al.*, 2007). Recently, tungsten-dependent enzymes were also found in aerobic bacteria, e.g. in *Methylobacterium extorquens* (Laukel *et al.*, 2003) or in *R. eutropha* (Cramm, 2009). In various studies it was shown that the interchange of molybdenum and tungsten led to inactive enzymes which can be exploited to identify whether the enzyme of interest is molybdenum- or tungsten-dependent (May *et al.*, 1988; McMaster & Enemark, 1998). However, for the N-formylmethanofuran dehydrogenases from *Methanobacterium thermoautotrophicum* and *Methanobacterium wolfeii* the interchange of molybdenum and tungsten yields enzymes with comparable activities (Johnson *et al.*, 1996). Our results show that the FDH from *C. glutamicum* ATCC 13032 is a molybdenum-dependent enzyme since the presence of tungstate almost completely inhibited formate consumption by *C. glutamicum* ATCC 13032 (Fig. 6).

In contrast with the deletion of cg0618 or cg0616, the deletion of cg0617 did not lead to the inability to consume formate, yet the consumption was strongly diminished. No putative conserved protein domain could be identified for Cg0617. However, although there is no significant homology to MobA from *E. coli*, cg0617 is annotated to encode a molybdopterin guanine dinucleotide biosynthesis protein which could be functionally identical to MobA of *E. coli* and *M. tuberculosis*. In contrast, the predicted protein encoded by cg1350 (*mob*) exhibits significant homology to MobA (27 % sequence identity) and it is possible that it partially complements *C. glutamicum* ATCC 13032 Δcg0617, explaining the lowered consumption of formate by reduced FDH activity. However, the detailed function of Cg0617 remains to be studied.

The ^{13}C-labelled formate experiment showed that formate is completely converted to $^{13}CO_2$ by *C. glutamicum* ATCC 13032 wild-type. The oxidation of formate to CO_2 generates two electrons transferred to an electron acceptor. We did not find evidence for FdhF being an integral membrane protein since protein sequence analysis tools did not find indications for signal peptides or transmembrane helices. Thus, FdhF from *C. glutamicum* ATCC 13032 appears to be a soluble protein in the cytosol or associated to the membrane. In enzyme assays with cell-free extracts, we could not detect formate dehydrogenase activity using NAD^+ or $NADP^+$ as cofactors (data not shown). In whole cell biotransformation processes, FDHs are frequently used for cofactor regeneration, like the NAD^+-dependent FDH from *Mycobacterium vaccae* (Bäumchen *et al.*, 2007; Litsanov *et al.*, 2012a). However, FDH from *C. glutamicum* ATCC 13032 is most likely not applicable for such processes and the oxidation of formate led to the generation of reducing equivalents, which are transferred to a currently unknown electron acceptor.

Well-characterized FDHs from various organisms participate in methylotrophic and chemoautrotrophic metabolism or are involved in anaerobic fermentative or respiratory processes. *C. glutamicum* ATCC 13032 does not contain the ability for either methylotrophic or chemoautotrophic growth. Furthermore, the FDH from *C. glutamicum* ATCC 13032 is active under oxic conditions. Also, oxidation of formate to CO_2 seemingly does not generate additional energy that could be used for growth. We speculate that the FDH in *C. glutamicum* ATCC 13032 is involved in the stress response. The growth of *C. glutamicum* ATCC 13032 is inhibited by the presence of formate, which is a natural component of soil, the habitat of *C. glutamicum* ATCC 13032. However, formate is mostly found only in micromolar amounts in the soil (Ahumada *et al.*, 2001). Possibly, the ability to inactivate (toxic) formate may confer a little growth advantage in the natural habitat. Putative FDHs with high sequence similarity to FdhF were found in a wide range of other soil bacteria, including *R. eutropha* and *Methylobacterium extorquens*, as well as in various corynebacteria, streptomyces, mycobacteria and rhodococci. These FDHs might fulfil functions in the stress response of the host and contribute to C1 turnover in syntrophic associations (Crable *et al.*, 2011).

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C_1 Metabolism in *Corynebacterium glutamicum*: an Endogenous Pathway for Oxidation of Methanol to Carbon Dioxide

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Methanol is considered an interesting carbon source in "bio-based" microbial production processes. Since *Corynebacterium glutamicum* is an important host in industrial biotechnology, in particular for amino acid production, we performed studies of the response of this organism to methanol. The *C. glutamicum* wild type was able to convert ^{13}C-labeled methanol to $^{13}CO_2$. Analysis of global gene expression in the presence of methanol revealed several genes of ethanol catabolism to be upregulated, indicating that some of the corresponding enzymes are involved in methanol oxidation. Indeed, a mutant lacking the alcohol dehydrogenase gene *adhA* showed a 62% reduced methanol consumption rate, indicating that AdhA is mainly responsible for methanol oxidation to formaldehyde. Further studies revealed that oxidation of formaldehyde to formate is catalyzed predominantly by two enzymes, the acetaldehyde dehydrogenase Ald and the mycothiol-dependent formaldehyde dehydrogenase AdhE. The Δ*ald* Δ*adhE* and Δ*ald* Δ*mshC* deletion mutants were severely impaired in their ability to oxidize formaldehyde, but residual methanol oxidation to CO_2 was still possible. The oxidation of formate to CO_2 is catalyzed by the formate dehydrogenase FdhF, recently identified by us. Similar to the case with ethanol, methanol catabolism is subject to carbon catabolite repression in the presence of glucose and is dependent on the transcriptional regulator RamA, which was previously shown to be essential for expression of *adhA* and *ald*. In conclusion, we were able to show that *C. glutamicum* possesses an endogenous pathway for methanol oxidation to CO_2 and to identify the enzymes and a transcriptional regulator involved in this pathway.

Methylotrophic microorganisms utilize reduced C_1 compounds, such as methane, methylamines, or methanol, as a sole carbon and energy source and play an important role in the global carbon cycle (1, 2). A key step in methylotrophic metabolism is the oxidation of methanol to formaldehyde. Whereas this oxidation step is catalyzed by flavin adenine dinucleotide (FAD)-dependent methanol oxidases with concomitant reduction of oxygen to hydrogen peroxide in methylotrophic yeast and fungi (3), Gram-negative methylotrophic bacteria, such as *Methylobacterium extorquens*, use pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenases to oxidize methanol in the periplasm (4). Gram-positive thermotolerant *Bacillus* strains usually contain NAD^+-dependent cytoplasmic methanol dehydrogenases (5). In methylotrophic metabolism, cytotoxic formaldehyde represents a crucial central metabolic intermediate. The toxicity of formaldehyde arises from nonenzymatic reactions with biological macromolecules such as proteins and DNA, leading to irreversible alkylations and cross-linkages (6–8). Formaldehyde detoxification can occur either by assimilation into cell material or by oxidation to carbon dioxide (9, 10). Pathways for formaldehyde oxidation to CO_2 are widely distributed and are not limited to methylotrophs, since virtually all organisms have to cope with toxic formaldehyde as a by-product of numerous environmental processes and various cellular demethylation and oxidation reactions. Hence, nature developed diverse strategies for its detoxification by efficient capture of formaldehyde by cofactors, such as glutathione, mycothiol, tetrahydrofolate, or tetrahydromethanopterin, and subsequent oxidation of the products (9, 10).

The soil bacterium *Corynebacterium glutamicum* can grow on various carbon sources, such as sugars, organic acids, alcohols, or aromatics, but presumably not on C_1 compounds since none of the known assimilation pathways is encoded in the genome. In agreement, it was reported that *C. glutamicum* strain R does not grow on methanol (11). In industrial biotechnology, highly productive strains of *C. glutamicum* serve to produce several million tons of amino acids annually, in particular the flavor enhancer L-glutamate and the feed additive L-lysine. In addition, strains have been developed for a variety of other commercially interesting compounds (12), such as organic acids (13–15), diamines (16–18), or alcohols (19–21). Moreover, *C. glutamicum* is also an efficient host for the production of heterologous proteins (see reference 22 and references therein).

Since methanol can be produced from renewable carbon sources (23), it is an interesting carbon source for "bio-based" production of chemicals or proteins. As a first step toward the goal of making *C. glutamicum* a methylotroph, we studied the response of this organism to methanol at the metabolic level and at the gene expression level. As a result, we describe the identification and characterization of an endogenous pathway for the oxidation of methanol to CO_2.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids used or constructed in the course of this work are listed in Table 1, and oligonucleotides are listed in in Table S1 in the supplemental material. *C. glutamicum* was routinely cultivated aerobically in 500-ml baffled shake flasks with 50 ml medium on a rotary shaker

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	F^- ϕ80d*lac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 phoA supE44* λ^- *gyrA96 relA1*	Invitrogen (Karslruhe, Germany)
C. glutamicum ATCC 13032	Biotin-auxotrophic wild-type strain	24
C. glutamicum Δ*fdhF* strain	Derivative of ATCC 13032 with in-frame deletion of *fdhF* gene	25
C. glutamicum Δ*adhA* strain	Derivative of ATCC 13032 with in-frame deletion of *adhA* gene	This work
C. glutamicum Δ*adhC* strain	Derivative of ATCC 13032 with in-frame deletion of *adhC* gene	This work
C. glutamicum Δ*adhE* strain	Derivative of ATCC 13032 with in-frame deletion of *adhE* gene	This work
C. glutamicum Δ*ald* strain	Derivative of ATCC 13032 with in-frame deletion of *ald* gene	This work
C. glutamicum Δcg2714 strain	Derivative of ATCC 13032 with in-frame deletion of cg2714	This work
C. glutamicum Δcg0273 strain	Derivative of ATCC 13032 with in-frame deletion of cg0273	This work
C. glutamicum Δ*ald* Δ*adhE* strain	Derivative of Δ*ald* strain with additional in-frame deletion of *adhE* gene	This work
C. glutamicum Δ*ald* Δcg0388 strain	Derivative of Δ*ald* strain with additional in-frame deletion of cg0388	This work
C. glutamicum Δ*ald* Δcg2193 strain	Derivative of Δ*ald* strain with additional in-frame deletion of cg2193	This work
C. glutamicum RG2	Derivative of ATCC 13032 with partial deletion of *ramA* gene	26
C. glutamicum Δ*mshC* strain	Derivative of ATCC 13032 with in-frame deletion of *mshC* gene	27
C. glutamicum Δ*ald* Δ*mshC* strain	Derivative of Δ*ald* strain with additional in-frame deletion of *mshC* gene	This study
C. glutamicum Δ*adhA*/pAN6-*adhA* strain	Plasmid-based complementation of *adhA* deletion	This work
C. glutamicum Δ*adhA*/pAN6 strain	Control strain	This work
C. glutamicum Δ*ald*/pAN6-*ald* strain	Plasmid-based complementation of *ald* deletion	This work
C. glutamicum Δ*adhE*/pAN6-*adhE* strain	Plasmid-based complementation of *adhE* deletion	This work
C. glutamicum Δ*ald* Δ*adhE*/pAN6-*ald-adhE* strain	Plasmid-based complementation of *ald-adhE* deletion	This work
Plasmids		
pK19*mobsacB*	Kan^r; mobilizable *E. coli* vector used for allelic exchange in *C. glutamicum* (pK18 $oriV_{E.c.}$ *oriT sacB lacZ*)	28
pK19Δ*adhA*	Kan^r; pK19*mobsacB* derivative containing 1,010-bp PCR product covering upstream and downstream regions of *adhA*	This work
pK19Δ*adhC*	Kan^r; pK19*mobsacB* derivative containing 966-bp PCR product covering upstream and downstream regions of *adhC*	This work
pK19Δ*ald*	Kan^r; pK19*mobsacB* derivative containing 1,017-bp PCR product covering upstream and downstream regions of *ald*	This work
pK19Δ*adhE*	Kan^r; pK19*mobsacB* derivative containing 1,042-bp PCR product covering upstream and downstream regions of *adhE*	This work
pK19Δcg0273	Kan^r; pK19*mobsacB* derivative containing 1,037-bp PCR product covering upstream and downstream regions of cg0273	This work
pK19Δcg2714	Kan^r; pK19*mobsacB* derivative containing 1,084-bp PCR product covering upstream and downstream regions of cg2714	This work
pK19Δcg0388	Kan^r; pK19*mobsacB* derivative containing 1,089-bp PCR product covering upstream and downstream regions of cg0388	This work
pK19Δcg2193	Kan^r; pK19*mobsacB* derivative containing 1,063-bp PCR product covering upstream and downstream regions of cg2193	This work
pK18*mobsacB*Δncgl1457	Kan^r; pK18*mobsacB* derivative containing PCR product covering upstream and downstream regions of ncgl1457 (*mshC*)	27
pAN6	Kan^r; *C. glutamicum*/*E. coli* shuttle vector; derivative of pEKEx2 (P*tac*, $lacI^q$, pBL1 $oriV_{C.g.}$, pUC18 $oriV_{E.c.}$)	29
pAN6-*adhA*	Kan^r; pAN6 derivative containing *adhA* under control of P*tac*	This work
pAN6-*ald*	Kan^r; pAN6 derivative containing *ald* under control of P*tac*	This work
pAN6-*adhE*	Kan^r; pAN6 derivative containing *adhE* under control of P*tac*	This work
pAN6-*ald-adhE*	Kan^r; pAN6 derivative containing *ald* and *adhE* under control of P*tac*	This work

(120 rpm) at 30°C. Either LB medium (30) or modified CGXII minimal medium (29) containing 28 to 222 mM glucose and/or 100 mM ethanol as a carbon and energy source was used. Methanol was added to the culture medium in concentrations from 50 mM to 3,000 mM using a 5 M stock solution. For strain construction and maintenance, either LB or BHIS agar plates (brain heart infusion [BHI] agar [Difco, Detroit, MI, USA] supplemented with 0.5 M sorbitol) were used. Media used in the course of gene deletion using pK19*mobsacB* were described before (31). *Escherichia coli* DH5α was used for cloning purposes and was grown aerobically on a rotary shaker (170 rpm) at 37°C in 5 ml LB medium or on LB agar plates (LB medium with 1.8% [wt/vol] agar). If appropriate, kanamycin was added to final concentrations of 25 μg ml^{-1} (*C. glutamicum*) or 50 μg ml^{-1} (*E. coli*). Growth was determined by measuring of the optical density at 600 nm (OD_{600}).

Recombinant DNA techniques. The enzymes for recombinant DNA work were obtained from Fermentas (St. Leon-Rot, Germany) and Merck Millipore (Billerica, Massachusetts, USA). Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared as described previously (32). *E. coli* was transformed by the RbCl method (33) and *C. glutamicum* ATCC 13032 by electroporation (34). Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (30). The oligonucleotides used for cloning were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1 in the supplemental material. The in-frame gene deletion mutants of *C. glutamicum* ATCC 13032 were constructed via a two-step homologous recombination procedure as described previously (31). The deletions in the chromosome were verified by PCR analysis using oligonucleotides hybridizing approximately 500 bp upstream and 500 bp downstream of the target gene (see Table S1). The coding regions of *adhA* (cg3107), *ald* (cg3096), and *adhE* (cg0387) were amplified by PCR using the oligonucleotide pairs P_*adhA*_fw_NdeI/P_*adhA*_rev_NheI, P_*ald*_fw_NdeI/P_*ald*_rev_NheI, and P_*adhE*_fw_NdeI/P_*adhE*_rev_NheI, respectively (see Table S1), digested with NdeI and NheI, and cloned into the expression vector pAN6 for complementation studies. The cloned regions were checked by DNA sequencing using plasmid-specific primers (see Table S1).

Determination of glucose and formate by HPLC. The concentrations of glucose and formate in cell-free culture supernatants were determined by HPLC analysis using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with a cation exchange column (organic acid refill column, 300 by 8 mm [CS-Chromatographie Service GmbH, Langerwehe, Germany]) as described previously (35).

Determination of methanol by gas chromatography. The quantitative detection of methanol in the cell-free supernatant of *C. glutamicum* cultures was performed via capillary gas chromatography (GC) using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany)] equipped with an HP-5 column [(5% phenyl)-methylpolysiloxane, 30 m, 0.32 mm, 0.25 μm (Agilent Technologies Waldbronn, Germany)]. Helium was used as a carrier gas with a flow rate of 2.8 ml/min. The sample (1 μl) was injected via split injection (1:50), employing a Combi PAL GC autosampler (CTC Analytics GmbH, Zwingen, Germany). The injector temperature was set to 250°C, and the column temperature was kept constant at 40°C. Detection was performed using a flame ionization detector (FID) at a detector temperature of 250°C. Calibration was performed with methanol as an external standard. Butanol (60 mM) was used as an internal standard and was added to the samples in the same volume ratio. Concentrations were calculated from peak areas using calibration with external methanol and internal butanol standards.

Determination of formaldehyde. Formaldehyde concentrations were determined via a colorimetric photometric assay as described by Nash (36). This assay is based on the condensation of acetylacetone and free formaldehyde in the presence of excess ammonium salt to the chromophore diacetyldihydrolutidine (Hanztsch reaction), which is characterized by an absorption maximum at 412 nm and an extinction coefficient of 7.7 mM^{-1} cm^{-1}. For the determination of formaldehyde concentrations, samples of 0.5 ml cell-free culture supernatant were taken at various time points during cultivation, mixed with 0.5 ml Nash reagent (2 M ammonium acetate, 50 mM acetic acid, and 20 mM acetylacetone), and incubated for 10 min at 60°C. Subsequently, the absorption at 412 nm was measured for each sample. A calibration curve showed that the assay was linear between 20 μM and 1.5 mM formaldehyde.

DNA microarray analysis. Whole-genome DNA microarray analyses were performed to monitor changes in the global gene expression of the *C. glutamicum* wild type in response to the presence of methanol. Cells of a preculture in LB medium were used to inoculate a second preculture in CGXII minimal medium with 111 mM glucose. To test the influence of methanol in the presence of glucose, two parallel main cultures in CGXII minimal medium with 111 mM glucose were inoculated with the second preculture to an OD_{600} of 0.5. After reaching an OD_{600} of 5, 100 mM methanol was added to one culture, whereas water was added to the second one as a control. After continuing incubation for 30 min, cells of both cultures were harvested in precooled (−20°C) ice-filled tubes via centrifugation (6,900 × *g*, 10 min, 4°C) and used for RNA isolation. To test the influence of methanol in the absence of glucose, a preculture in CGXII medium with 111 mM glucose was incubated until an OD_{600} of 8 was reached. Cells were washed with CGXII minimal medium without a carbon source and resuspended in this medium to an OD_{600} of 5. The cell suspensions were incubated for 2 h, and then 100 mM methanol was added to one of the suspensions and water to the second one. After incubation for another 30 min, cells were harvested and used for RNA isolation.

RNA isolation and synthesis of fluorescently labeled cDNA were carried out as described previously (37). For cDNA synthesis, 25 μg total RNA of each sample was used. Custom-made DNA microarrays for *C. glutamicum* ATCC 13032 printed with 70-er oligonucleotides were obtained from Operon (Cologne, Germany) and were based on the genome sequence entry NC_006958 (38). Processing of DNA microarrays and evaluation of the obtained data were performed as previously described (29). The normalized and processed data were saved in the in-house microarray database (39). All DNA microarray experiments were performed in three replicates.

Determination of alcohol dehydrogenase activities in crude extracts. For the determination of alcohol dehydrogenase activity *in vitro*, the *C. glutamicum* wild type was cultivated in CGXII minimal medium either with 56 mM glucose, with 56 mM glucose and 100 mM ethanol, or with 56 mM glucose and 100 mM methanol. Cells were grown until the early stationary phase (OD_{600} of ~22), and 50-ml culture samples were harvested by centrifugation for 15 min at 4,500 × *g* and 4°C. After washing in 25 ml buffer (100 mM Tris-HCl and 30% [vol/vol] glycerol, pH 7.5), cells were resuspended in 2 ml 100 mM Tris-HCl, 30% (vol/vol) glycerol, and 1 mM dithiothreitol, pH 7.5. Cells were disrupted by sonication (6 min; cycle, 0.5; amplitude, 50) using a UP200S sonifier (Hielscher GmbH, Stuttgart, Germany) with cooling on ice. After centrifugation (30 min, 16,000 × *g*, 4°C), the supernatant was used for enzyme assays. The protein concentration of the cell extracts was determined by the method of Bradford (40) using bovine serum albumin as the standard. The assay was performed as described previously (11) with slight modifications. The assay mixtures (1 ml total volume) contained 50 mM Tris-HCl (pH 8.6), 4 mM NAD^+, and 10 μl cell extract. The reaction was initiated by the addition of 340 mM ethanol and monitored by following the increase in absorption at 340 nm over 2 min using an Ultraspec 3100pro photometer (GE Healthcare Life Sciences, Freiburg, Germany) at 30°C. One unit of alcohol dehydrogenase activity is defined as the reduction of 1 μmol NAD^+ per minute.

Determination of $^{13}CO_2$ and $^{12}CO_2$. The conversion of methanol to CO_2 by *C. glutamicum* was monitored in ^{13}C labeling experiments. Cells were grown in 200 ml CGXII medium with 56 mM glucose in a Dasgip parallel bioreactor system (Dasgip AG, Jülich, Germany), and 100 mM ^{13}C-labeled methanol (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium immediately before inoculation. During cultivation, pH, dissolved oxygen concentration, and off-gas (CO_2 and O_2) were monitored online via the Dasgip monitoring system (Dasgip Control 4.0). Whereas the pH was not controlled during cultivation, the dissolved O_2 saturation was kept at >30%, varying the stirrer speed between 400 and 1,200 rpm at a constant airflow rate of 4 standard liters (sl)/h. If required, antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) was added. Growth was monitored offline by OD_{600} measurements. $^{13}CO_2$ and $^{12}CO_2$ off-gas analysis and quantification were performed using an FT-IR gas analyzer (Gasmet CR-2000i [Ansyco, Karlsruhe, Germany]) and the Calcmet software program, version 10 (Gasmet Technologies Oy, Helsinki, Finland).

Microarray data accession number. The microarray data were deposited in the GEO database with the accession number GSE49936.

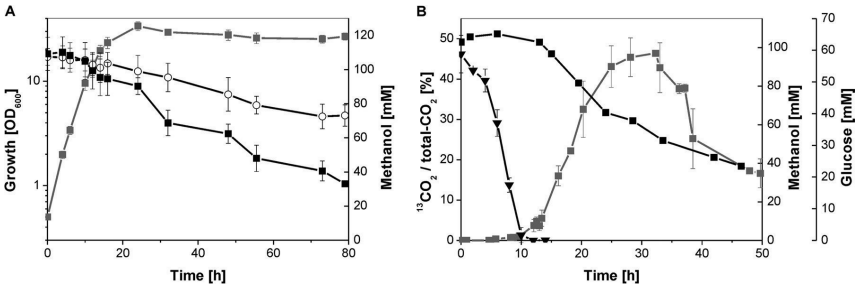

FIG 1 (A) Methanol consumption (■) and growth (■, in gray) of the *C. glutamicum* wild type in CGXII minimal medium supplemented with 111 mM glucose and 120 mM methanol. Four independent experiments were performed in shake flasks, always accompanied by a methanol evaporation control without cells (○). (B) $^{13}CO_2$ formation as $^{13}CO_2$/total CO_2 ratio (■, in gray), methanol consumption (■), and glucose consumption (▼) during growth of the *C. glutamicum* wild type in bioreactors with 56 mM glucose in the presence of 100 mM ^{13}C-labeled methanol. The *C. glutamicum* wild type showed a growth rate of 0.27 ± 0.01 h^{-1} and a final OD_{600} of 20.4 ± 1.8.

RESULTS

Methanol is metabolized by *C. glutamicum* in stationary growth phase. Initial cultivation experiments of the *C. glutamicum* wild type in CGXII minimal medium containing 111 mM glucose and methanol at concentrations ranging from 0.05 M to 3 M showed that the growth rate (μ) decreased with increasing methanol concentrations. The following values were obtained: 0.41 h^{-1} at 0 mM, 0.33 h^{-1} at 0.05 M, 0.28 h^{-1} at 0.1 M, 0.24 h^{-1} at 0.2 M, 0.21 h^{-1} at 0.7 M, 0.19 h^{-1} at 1 M, 0.17 h^{-1} at 1.3 M, and 0.09 h^{-1} at 1.5 M (see Fig. S1 in the supplemental material). At 3 M methanol, growth was almost completely inhibited. Cultivation experiments in minimal medium with 111 mM glucose and 120 mM methanol revealed that the *C. glutamicum* wild type consumes methanol against the background of methanol evaporation with a rate of 63.2 μmol h^{-1} (g cell dry weight [CDW])$^{-1}$ (Fig. 1A). After 72 h of cultivation, only 41 mM methanol remained in the minimal medium, whereas 73 mM methanol could be detected in the control flask without *C. glutamicum* cells. Since *C. glutamicum* was not able to grow on methanol as a sole carbon source and does not possess one of the known pathways for methanol assimilation (data not shown), we assumed that methanol is oxidized to CO_2. To test this assumption, cultivation experiments were performed in bioreactors using CGXII minimal medium with 56 mM glucose and 100 mM ^{13}C-labeled methanol. During these experiments, ^{13}C-labeled CO_2 was detected by Fourier transform infrared spectroscopy (FTIR) as soon as methanol consumption started in the stationary growth phase, when glucose had been completely consumed (Fig. 1B).

FdhF is responsible for oxidation of formate to carbon dioxide. Oxidation of methanol to CO_2 is catalyzed stepwise by specific dehydrogenases via the intermediates formaldehyde and formate (10). Whereas no genes coding for methanol-or formaldehyde dehydrogenase have been annotated in the genome of *C. glutamicum* (41, 42), we recently identified genes coding for a molybdenum-containing formate dehydrogenase (25). To test the involvement of this enzyme in methanol oxidation, growth experiments similar to those described for Fig. 1A were conducted with a *C. glutamicum* Δ*fdhF* mutant (25), which resulted in a slightly reduced methanol consumption (27 mM ± 4 mM) compared to that of the wild type (34 mM ± 6 mM) after 72 h against an evaporation background. Most important, the mutant formed an equimolar concentration of formate (27 mM ± 2 mM) in the culture supernatant, whereas the wild type formed only 18 mM ± 4 mM. This result shows that FdhF is responsible for formate oxidation to CO_2 in the wild type and confirms that methanol or formaldehyde is not assimilated by *C. glutamicum*. Additionally, the formate accumulation during growth of the wild type in methanol-containing medium indicated that the oxidation of formate to CO_2 is the limiting factor in the endogenous methanol oxidation pathway in *C. glutamicum*.

Search for genes involved in methanol oxidation to CO_2 by global gene expression analysis. In order to identify genes that might be involved in the oxidation of methanol and formaldehyde, we tested the influence of methanol on global gene expression using DNA microarray analysis. Gene expression was considered to be influenced by methanol if mRNA levels in at least two out of three biological replicates differed from those of the control without methanol. In total, 20 genes were up- and 19 genes were downregulated in the presence of 100 mM methanol (see Table S2 in the supplemental material). Interestingly, two genes known to be essential for the oxidation of ethanol to acetate in *C. glutamicum* were among the upregulated genes: *adhA* (cg3107; 4.4-fold), encoding an alcohol dehydrogenase (11, 43), and *ald* (cg3096; 2.1-fold), encoding acetaldehyde dehydrogenase (44).

Besides *adhA*, four other genes have been annotated as putative alcohol dehydrogenase genes in the genome of *C. glutamicum* (43): cg0273, cg0387 (*adhE*), cg0400 (*adhC*), and cg2714. Their expression levels were not altered in the presence of methanol. To determine the relevance of *adhA*, *ald*, and the other putative alcohol dehydrogenase genes for methanol or formaldehyde oxidation in *C. glutamicum*, in-frame deletion mutants lacking these genes were constructed and characterized.

AdhA is mainly responsible for oxidation of methanol to formaldehyde. The Δ*adhA*, Δ*adhC*, Δ*adhE*, Δcg2714, and Δcg0273 *C. glutamicum* deletion mutants were analyzed regarding their ability to oxidize methanol. All deletion mutants showed the same growth behavior in CGXII medium containing 111 mM glucose and 120 mM methanol (see Fig. S2 in the supplemental material). However, whereas the methanol oxidation rate of the Δ*adhC*, Δ*adhE*, Δcg2714, and Δcg0273 strains (70.0 ± 20, 73 ± 4, 70 ± 4,

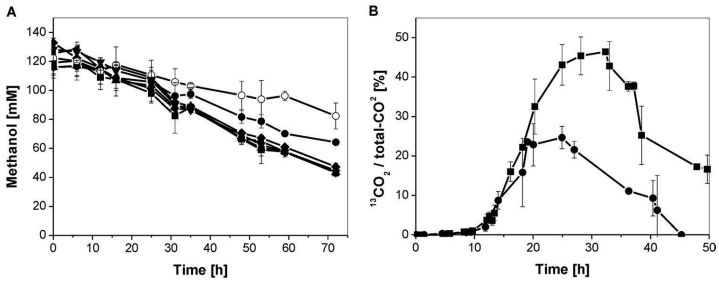

FIG 2 (A) Methanol oxidation of *C. glutamicum* wild type (■), Δ*adhA* strain (●), Δ*adhC* strain (▲), Δ*adhE* strain (▼), Δcg2714 strain (◆), and Δcg0273 strain (◀) in CGXII minimal medium supplemented with 111 mM glucose and 120 mM methanol. Two to four independent experiments in shake flasks were performed, always accompanied by a methanol evaporation control (○). (B) $^{13}CO_2$ formation as $^{13}CO_2$/total CO_2 ratio of *C. glutamicum* wild type (■) and Δ*adhA* strain (●) during cultivation in bioreactors in the presence of 100 mM ^{13}C-labeled methanol. Experiments to determine $^{13}CO_2$ generation in the *C. glutamicum* wild type were performed in triplicate and those for the Δ*adhA* mutant in duplicate. Mean values are shown.

and 61 ± 3 μmol h^{-1} [g CDW]$^{-1}$, respectively) were similar to that of the wild type (63.3 ± 10 μmol h^{-1} [g CDW]$^{-1}$), the rate for the Δ*adhA* mutant was reduced by about 62%, to 26.1 ± 6.1 μmol h^{-1} (g CDW)$^{-1}$ (Fig. 2A). Complementation of the Δ*adhA* strain with the expression plasmid pAN6-*adhA* (plus 1.5 mM isopropyl-β-D-thiogalactopyranoside [IPTG]) restored the wild-type methanol oxidation rate (data not shown). [^{13}C]Methanol labeling experiments confirmed the participation of AdhA in methanol oxidation, since the Δ*adhA* mutant generated 60% less $^{13}CO_2$ than the wild type (Fig. 2B). These results indicate that the first step in methanol oxidation by *C. glutamicum* is predominantly catalyzed by AdhA but that at least one additional dehydrogenase of hitherto unknown identity is also involved.

Enzyme assays with *C. glutamicum* crude extracts revealed that cells cultivated in minimal medium with 56 mM glucose and 100 mM methanol possessed a 3-fold higher alcohol dehydrogenase activity (280 ± 22 U/g protein) in stationary phase than cells grown solely with 56 mM glucose (96 ± 60 U/g), suggesting that *adhA* expression is induced by methanol. Previous studies with *C. glutamicum* strain R are in agreement with this result (11).

Ald oxidizes formaldehyde to formate. In preliminary experiments, we tested growth of the *C. glutamicum* wild type in the presence of various concentrations of formaldehyde. In the presence of 2 mM formaldehyde, it showed a slight growth inhibition, and it showed a strong growth defect at 4 mM formaldehyde (see Fig. S3 in the supplemental material). As displayed in Fig. 3A, growth of the Δ*ald* mutant was slightly more inhibited by 2 mM formaldehyde than growth of the wild type, indicating a decreased ability of the mutant to metabolize formaldehyde. This was confirmed by the finding that the rate of formaldehyde disappearance as measured by the Nash assay was only 0.66 ± 0.1 mM formaldehyde h^{-1} for the Δ*ald* mutant, but it was 1.17 mM ± 0.18 mM h^{-1} for the wild type (Fig. 3B). Complementation of the Δ*ald* mutant with the expression plasmid pAN6-*ald* (plus 0.3 mM IPTG) led to a formaldehyde oxidation rate of 2.2 mM ± 0.3 mM h^{-1}. These results clearly indicate that Ald plays an important role in formaldehyde oxidation to formate but that another enzyme involved in formaldehyde consumption must be present in *C. glutamicum*.

Mycothiol-dependent oxidation of formaldehyde via AdhE. Inspection of the genome sequence of *C. glutamicum* for further candidates involved in formaldehyde oxidation led to the identification of Cg0387 (AdhE). As mentioned above, AdhE is annotated as alcohol dehydrogenase but shares 66 to 69% amino acid sequence identity to mycothiol-dependent formaldehyde dehydrogenases of *Amycolatopsis methanolica* (45), *Mycobacterium*

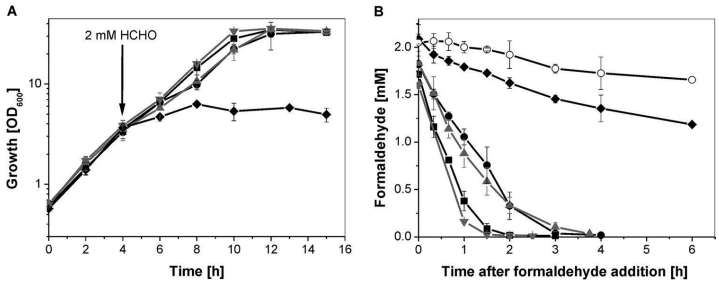

FIG 3 Growth(A) or formaldehyde oxidation (B) of *C. glutamicum* wild type (■) and Δ*ald* (●), Δ*adhE* (▲, in gray), Δcg0273 (▼, in gray), and Δ*ald* Δ*adhE* (◆) mutant strains in CGXII minimal medium with 111 mM glucose. After cultivation for 4 h, 2 mM formaldehyde was added (indicated by an arrow in panel A). Mean values and standard deviations for three biological replicates are shown, always accompanied by an evaporation control (○).

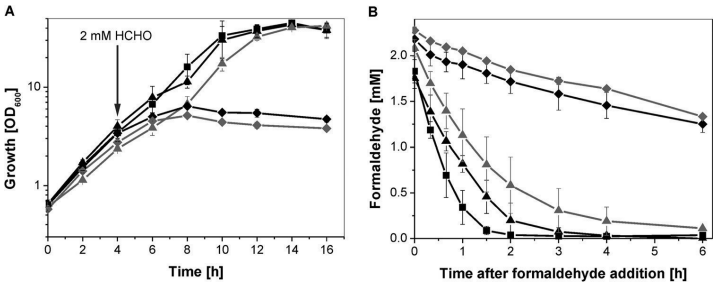

FIG 4 Growth (A) or formaldehyde oxidation (B) of *C. glutamicum* wild type (■) and Δ*adhE* (▲), Δ*mshC* (▲, in gray), Δ*ald* Δ*adhE* (◆), and Δ*ald*Δ*mshC* (◆, in gray) mutant strains in CGXII minimal medium with 111 mM glucose. After cultivation for 4 h, 2 mM formaldehyde was added (indicated by an arrow in panel A). Mean values and standard deviations for three biological replicates are shown.

smegmatis (46), and *Rhodococcus erythropolis* (47). These proteins catalyze the NAD^+-dependent oxidation of a spontaneously formed product of mycothiol and formaldehyde, *S*-hydroxymethyl-mycothiol, to *S*-formyl-mycothiol, which can subsequently be hydrolyzed to formate and mycothiol (48–50). Similar to the case with the *C. glutamicum* Δ*ald* strain, the deletion of *adhE* resulted in slightly impaired growth in the presence of 2 mM formaldehyde (Fig. 3A), and the rate of formaldehyde disappearance was reduced to 0.74 ± 0.06 mM h^{-1}, compared to 1.17 mM ± 0.18 mM h^{-1} observed for the wild type (Fig. 3B). Complementation of the Δ*adhE* mutant with the expression plasmid pAN6-*adhE* (plus 0.3 mM IPTG) led to a formaldehyde oxidation rate of 1.7 mM ± 0.7 mM h^{-1}. Thus, besides Ald, AdhE is also involved in formaldehyde oxidation in *C. glutamicum*.

To test for additional formaldehyde oxidation enzymes, the *C. glutamicum* Δ*ald* Δ*adhE* double mutant was constructed and characterized. As shown in Fig. 3A, growth of the Δ*ald* Δ*adhE* strain stopped shortly after addition of 2 mM formaldehyde to the minimal medium, and formaldehyde consumption was almost completely prevented (Fig. 3B). Plasmid-based expression of *ald* and *adhE* in the *C. glutamicum* Δ*ald* Δ*adhE* strain with pAN6-*ald-adhE* (plus 0.3 mM IPTG) restored the ability to oxidize formaldehyde (2.3 mM ± 0.1 mM h^{-1}). [^{13}C]Methanol labeling experiments further confirmed the participation of Ald and AdhE in the methanol oxidation pathway, since the Δ*ald* Δ*adhE* strain generated 75% less $^{13}CO_2$/total CO_2 than the wild type (data not shown). However, $^{13}CO_2$ was still generated, and although the methanol oxidation rate of the Δ*ald* Δ*adhE* strain was lower than that of the wild type (32.9 ± 3.3 μmol h^{-1} [g CDW]$^{-1}$ versus 63.3 ± 10 μmol h^{-1} [g CDW]$^{-1}$), *C. glutamicum* possesses another possibility for detoxifying formaldehyde besides using Ald and AdhE.

To verify the mycothiol-dependence of AdhE in *C. glutamicum*, a strain deficient in mycothiol biosynthesis (*C. glutamicum* Δ*mshC* [27]) was investigated regarding its capability to oxidize formaldehyde. The deletion of *mshC* resulted in an impaired growth in the presence of 2 mM formaldehyde (Fig. 4A), and similar to the case with the *C. glutamicum* Δ*adhE* strain, the formaldehyde oxidation rate was reduced (0.54 mM ± 0.11 mM h^{-1}) in comparison to that of the wild type (1.7 mM ± 0.7 mM h^{-1}) (Fig. 4B). In addition, the *C. glutamicum* Δ*ald* Δ*mshC* double deletion mutant was constructed. It showed a phenotype similar to that of the *C. glutamicum* Δ*ald* Δ*adhE* strain, with respect to growth and its capability to oxidize formaldehyde; growth stopped shortly after addition of 2 mM formaldehyde to the minimal medium, and formaldehyde consumption was almost completely prevented (Fig. 4B). These results clearly support that formaldehyde oxidation by AdhE is strictly dependent on mycothiol.

Three possible pathways for conversion of *S*-formyl-mycothiol were reported: spontaneous hydrolysis to formate and mycothiol, hydrolysis to formate and mycothiol by an *S*-formyl-mycothiol hydrolase (FMH), or oxidation by a molybdoprotein aldehyde dehydrogenase to *S*-carboxy-mycothiol, which spontaneously decomposes to carbon dioxide and mycothiol (46, 51). The latter possibility appears unlikely for *C. glutamicum*, since the Δ*fdhF* mutant converted methanol stoichiometrically to formate. The gene located immediately downstream of *adhE* in *C. glutamicum*, cg0388, is annotated as Zn-dependent hydrolase, and the derived protein sequence has 65% sequence identity to that of the proposed *R. erythropolis* FMH (47). In addition, the putative lysophospholipase (cg2193) shows 38% sequence identity to the *Mycobacterium tuberculosis* Rv2854 protein, which is expected to be an FMH. It is located in an operon with the NADPH-dependent mycothiol reductase gene *mtr*, and the organization of these genes is conserved in most mycobacteria and corynebacteria, as well as in *C. glutamicum* (49).

To test the role of the genes cg0388 and cg2193 in methanol oxidation, both genes were individually deleted in the *C. glutamicum* Δ*ald* background. Growth experiments in CGXII minimal medium containing 56 mM glucose and 120 mM methanol revealed that the *C. glutamicum* Δ*ald* Δcg0388 strain and the *C. glutamicum* Δ*ald* Δcg2193 strains accumulate as much formate (15.1 ± 0.5 mM and 17.5 ± 0.5 mM, respectively) within 60 h as the wild type (16.7 ± 0.4 mM) and significantly more than the Δ*ald* Δ*adhE* mutant (6.1 ± 0.7 mM), suggesting that neither Cg0388 nor Cg2193 is essential for conversion of *S*-formyl-mycothiol to mycothiol and formate, but this does not exclude that they possess such an activity. These results hint at a spontaneous hydrolysis of *S*-formyl-mycothiol to formate and mycothiol.

Methanol consumption by *C. glutamicum* is subject to catabolite repression. As shown above (Fig. 1), methanol oxidation is subject to carbon catabolite repression by glucose, since it starts in the stationary growth phase after glucose has been consumed.

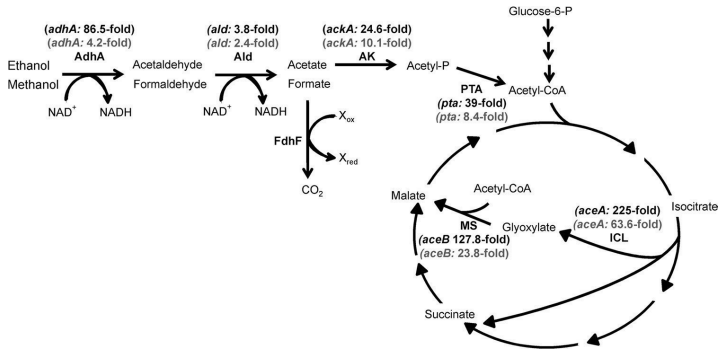

FIG 5 Influence of methanol on expression of selected genes in *C. glutamicum*. DNA microarray experiments showed that genes participating in ethanol catabolism and methanol oxidation are upregulated in the presence of methanol (AdhA, alcohol dehydrogenase; Ald, acetaldehyde dehydrogenase; AK, acetate kinase; PTA, phosphotransacetylase; MS, malate synthase; ICL, isocitrate lyase). The influence of methanol was more pronounced when methanol was added to resting cells in the absence of glucose (black numbering) than after the addition of methanol to cells grown with glucose (gray numbers).

Such a behavior is quite unusual for *C. glutamicum*, since the majority of carbon sources, such as acetate (52) and gluconate (29), are consumed in parallel by this bacterium. Two known exceptions are glutamate (53) and ethanol (11, 54). In the case of ethanol, *adhA* expression was shown to be subject to a complex transcriptional control involving RamA as an essential activator and RamB and SucR as repressors (43, 55). RamA and RamB are global transcriptional regulators in *C. glutamicum* and control genes for enzymes of the central metabolism (26, 56, 57). SucR is now termed AtlR (58). In the case of *ald*, RamA was described as an essential activator and RamB as a weak repressor (44). Since AdhA was shown to be the major enzyme responsible for methanol oxidation to formaldehyde, we tested methanol consumption by a *C. glutamicum* Δ*ramA* mutant (26) and observed that its capability to oxidize methanol was strongly reduced (see Fig. S4A in the supplemental material) and was comparable to that of a Δ*adhA* mutant. Further support for the methanol oxidation defect of the Δ*ramA* mutant was obtained by [^{13}C]methanol labeling experiments. They revealed that the Δ*ramA* mutant produced 86% less $^{13}CO_2$/total CO_2 than the wild type (see Fig. S4B). These results are in agreement with the essential role of RamA for *adhA* expression.

To determine the methanol oxidation capacity of *C. glutamicum* under optimal conditions, i.e., in the absence of carbon catabolite repression (which in the case of *adhA* is seen not only with glucose but also with acetate [43]), we analyzed cells cultivated in CGXII minimal medium containing 100 mM methanol and 100 mM ethanol. Under these conditions, the specific methanol consumption rate was 366 ± 19 μmol h^{-1} (g CDW)$^{-1}$, compared to 170 ± 60 μmol h^{-1} (g CDW)$^{-1}$ in medium containing 100 mM methanol and 28 mM glucose.

The DNA microarray experiments reported above were performed with cells cultivated in the presence of 111 mM glucose and 100 mM methanol. To test whether methanol alone provokes a more pronounced up- or downregulation of relevant genes, another set of DNA microarray experiments was performed in which RNA was prepared from resting cells incubated with or without methanol as a sole carbon source. In this case, an about 5-fold-higher expression of genes involved in methanol, ethanol, and acetate metabolism was observed than in the previous DNA microarray experiment, confirming the negative effect of glucose on these genes (Fig. 5). The total number of genes showing altered expression in these experiments was much larger due to the usage of resting cells (see Table S3 in the supplemental material).

DISCUSSION

In this study, the capability of *C. glutamicum* for oxidation of methanol to CO_2 was demonstrated for the first time, and the key enzymes involved in this endogenous pathway were identified (Fig. 6). The dissimilation of C_1 compounds is widespread in nature and has also been found in other members of the *Actinomycetales*, such as *Amycolatopsis methanolica*, *R. erythropolis*, or *M. smegmatis* (59–61).

Our results revealed that the NAD^+- and zinc-dependent alcohol dehydrogenase AdhA is primarily responsible for methanol oxidation. This is in line with the finding that AdhA of *C. glutamicum* strain R, which has 98% sequence identity to AdhA of strain ATCC 13032, is active not only with ethanol but also with methanol, propanol, and butanol (11). Besides AdhA, one or more additional dehydrogenase(s) capable of oxidizing methanol must be present in *C. glutamicum*. In contrast to the Δ*adhA* strain, the Δ*adhC*, Δ*adhE*, Δcg2714, and Δcg0273 deletion mutants did not show a reduction of the methanol oxidation rate, arguing against an important role of the encoded putative alcohol dehydrogenases in methanol catabolism.

The oxidation of formaldehyde by *C. glutamicum* was shown to be predominantly catalyzed by the enzymes Ald (Cg3096) and AdhE (Cg0387). Similar to AdhA, Ald is essential for growth on ethanol, since it catalyzed the oxidation of acetaldehyde to acetate (44). Interestingly, Ald of *C. glutamicum* shows 70% sequence identity to an NAD^+-dependent aldehyde dehydrogenase (AldhR) from *R. erythropolis* UPV-1 (62). This enzyme is characterized by broad substrate specificity for aliphatic aldehydes, such as *n*-hexanal, *n*-octanal, and formaldehyde. Hence, it is no surprise that Ald of *C. glutamicum* also accepts formaldehyde as the substrate.

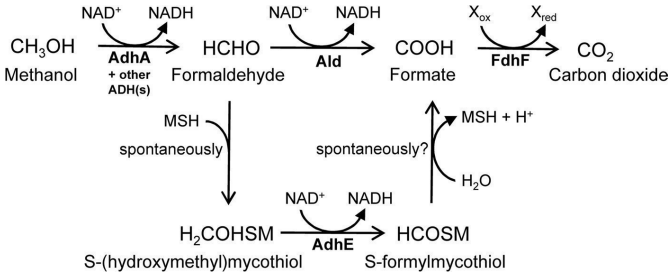

FIG 6 Overview of the methanol oxidation pathway in *C. glutamicum*. Methanol is oxidized to formaldehyde by the alcohol dehydrogenase AdhA and at least one additional unknown dehydrogenase. Formaldehyde is then either directly oxidized to formate by the acetaldehyde dehydrogenase Ald or spontaneously forms *S*-(hydroxymethyl)mycothiol with mycothiol (MSH). Subsequently, AdhE oxidizes *S*-(hydroxymethyl)mycothiol to *S*-formylmycothiol, which hydrolyzes to mycothiol and formate. Finally, formate is oxidized to carbon dioxide by the formate dehydrogenase FdhF, which is dependent on a yet-unknown redox cofactor.

Sequence similarity analyses revealed that AdhE belongs to the class III zinc-dependent alcohol dehydrogenases but also exhibits high sequence identity to the MSH-dependent formaldehyde dehydrogenase AdhC of *R. erythropolis*. In this organism, AdhC oxidizes aliphatic alcohols ranging from ethanol to octanol but not methanol in an MSH-independent manner in addition to the MSH-dependent oxidation of formaldehyde (60). The mycothiol dependence of formaldehyde oxidation by AdhE of *C. glutamicum* was confirmed by the fact that a mycothiol-defective mutant (Δ*mshC*) showed the same phenotype as a Δ*adhE* mutant and a Δ*ald* Δ*mshC* double mutant behaved similarly to a Δ*ald* Δ*adhE* mutant. In *C. glutamicum*, mycothiol was reported to contribute to resistance to alkylating agents, glyphosate, ethanol, antibiotics, heavy metals, and aromatic compounds (63). Our findings indicate that *C. glutamicum*, similar to *R. erythropolis* (47), can oxidize formaldehyde in an MSH-dependent manner via AdhE and in an NAD-dependent manner via Ald.

In the case of MSH-dependent oxidation of formaldehyde, the intermediate *S*-formylmycothiol is formed. In *M. tuberculosis*, a gene (Rv2854) is found which might code for an *S*-formylmycothiol hydrolase (FMH). However, no thiol esterase activity converting *S*-formylmycothiol to formate was detected in *M. tuberculosis*, suggesting that *S*-formylmycothiol might be oxidatively converted by a molybdoprotein aldehyde dehydrogenase to an unstable carbon dioxide ester, which then spontaneously decomposes to CO_2 and MSH (51). A *C. glutamicum* Δ*ald* mutant still accumulates the same amount of formate as the wild type, indicating that *S*-formylmycothiol generated by AdhE is not oxidized to CO_2 but is hydrolyzed to formate and mycothiol either spontaneously or by an FMH. The genes cg0388 and cg2193 show sequence similarity to putative FMHs of *R. erythropolis* and *M. tuberculosis*. However, the Δ*ald* Δcg0388 and Δ*ald* Δcg2193 double mutants were unaltered with respect to formate accumulation, indicating that the Cg0388 and Cg2193 proteins are not required for hydrolysis of *S*-formyl-mycothiol and that this step may occur spontaneously.

The last step in the methanol oxidation pathway, the oxidation of formate to CO_2, was shown to be catalyzed by the previously identified molybdenum cofactor-dependent formate dehydrogenase FdhF (25). The electron acceptor of this enzyme has not yet been identified.

During this study, we observed that methanol oxidation in *C. glutamicum* is subject to catabolite repression by glucose. Repression of *adhA* and *ald* expression by glucose has been described first for studies of ethanol catabolism in *C. glutamicum* (44). Expression of both genes is strictly dependent on the transcriptional regulator RamA, since a *ramA*-deficient strain was no longer able to grow on ethanol as a carbon source (26, 44, 54, 56). Our DNA microarray experiments confirmed the upregulation of *adhA* and *ald* in the presence of methanol, and enzyme assays with cell extracts revealed that the NAD-dependent ethanol dehydrogenase activity of cells cultivated in minimal medium with glucose and methanol was 3-fold higher than that in cells grown solely with glucose. Thus, not only ethanol but also methanol apparently is capable of triggering transcription of RamA-activated genes. The deletion mutant *C. glutamicum* Δ*ramA* was strongly impaired in its ability to oxidize methanol, in line with the fact that RamA is essential for transcriptional activation of *adhA* and *ald*. Until now, it has not been clear how RamA activity is controlled at the protein level. Our results clearly indicate that not only C_2 metabolites but also C_1 metabolites are capable of activating RamA.

In summary, we identified four enzymes (AdhA, Ald, AdhE, and Fdh) and one transcriptional regulator (RamA) that are involved in the endogenous oxidation of methanol to CO_2 by *C. glutamicum*. As indicated in the introduction, methanol could be an interesting carbon source for the microbial production of food and feed additives or fine chemicals (23). To date, only a few methylotrophic bacteria, such as *Bacillus methanolicus* or *Methylophilus methylotrophus*, were engineered for the production of amino acids from methanol (64, 65). As an alternative approach, it might be possible to establish the ability to utilize methanol as a carbon source in naturally nonmethylotrophic *C. glutamicum* production strains by introducing suitable heterologous pathways, such as the ribulose monophosphate pathway or the serine pathway. The elucidation of the route for methanol dissimilation to carbon dioxide and its regulation achieved in this study represents a first step toward this goal.

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3.3

Metabolic Engineering of *Corynebacterium glutamicum* for the Metabolization of Methanol

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In the chemical industry, methanol is already an important carbon feedstock, but in biotechnology, it has found only limited application. This can mostly be attributed to the inability of microbial platform organisms to utilize methanol as carbon and energy source. With the aim to turn methanol into a suitable feedstock for microbial production processes, we engineered the industrially important, but non-methylotrophic bacterium *Corynebacterium glutamicum* towards the utilization of methanol as auxiliary carbon source in a sugar-based medium. Initial oxidation of methanol to formaldehyde was achieved by heterologous expression of a methanol dehydrogenase from *Bacillus methanolicus* whereas assimilation of formaldehyde was realized by implementing the key enzymes of the ribulose monophosphate pathway from *B. subtilis*: 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase. The recombinant *C. glutamicum* strain showed an average methanol consumption rate of 1.7 ± 0.3 mM/h in a glucose/methanol growth medium and the culture grew to a higher cell density as compared to medium without methanol. In addition, ^{13}C-methanol labeling experiments revealed labeling fractions of 3-10% in the m+1 mass isotopomers of various intracellular metabolites. In the background of a *C. glutamicum* Δ*ald*Δ*adhE* mutant, which is strongly impaired in its ability to oxidize formaldehyde to CO_2, the m+1 labeling of these intermediates was even higher (8-25%) pointing to an increased methanol assimilation flux in this strain. The engineered strains represent a promising starting point for amino acid production with *C. glutamicum* using methanol as auxiliary substrate during sugar-based processes.

Highly productive strains of *Corynebacterium glutamicum* have been developed to produce several million tons of amino acids annually, in particular the feed additive L-lysine and the flavor enhancer L-glutamate. In addition, extensive research has focused on engineering *C. glutamicum* for the microbial production of a variety of other commercially interesting compounds (1), such as organic acids (2-4), diamines (5-7), or alcohols (8-10). The natural substrate spectrum of *C. glutamicum* includes sugars, organic acids or alcohols, but for industrial production processes, mainly glucose (starch) or sucrose and fructose (molasses) are used as carbon sources (11-13). The availability and price of sugar is dependent from seasonal variations and weather conditions as well as on price regulations and import limitations imposed on agricultural products. This leads to strong fluctuations in the sugar price and due to the increasing world population and loss of arable land it is even expected to rise in the coming decades. Thus, the

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demand for an alternative carbon source for the microbial production of small compounds arises (14). Over the past years, *C. glutamicum* was already genetically engineered towards the ability to efficiently utilize several cheap carbon sources, e.g. the TCA cycle intermediates malate, fumarate and succinate, the lignocellulose compounds arabinose and xylose as well as starch, cellobiose, glycerol, lactose, galactose and glucoseamine [(11, 15) and references within]. In addition, methanol represents an interesting alternative, since it is a pure and homogenous raw material and due to its low price it is already an important carbon feedstock in the chemical industry. At present, methanol is mainly produced from synthesis gas (a mixture of CO and H_2), which is obtained by catalytic reforming of coal or natural gas. However, approaches exist to produce it from renewable carbon sources (16-19). Availability and market price of methanol raises the question if this C_1-compound could serve as alternative carbon source for microbial production processes (20, 21). Although *C. glutamicum* harbors an endogenous pathway for oxidation of methanol to CO_2 (22, 23), it is a non-methylotrophic organism and not able to utilize C_1 compounds as sole carbon and energy source.

A key step in the methylotrophic metabolism is the oxidation of methanol to formaldehyde. Whereas Gram-negative methylotrophic bacteria, such as *Methylobacterium extorquens*, employ pyrroloquinoline quinone (PQQ)-dependent, periplasmic methanol dehydrogenases to oxidize methanol (24), Gram-positive thermotolerant *Bacillus* strains usually use NAD^+-dependent cytoplasmic methanol dehydrogenases (25). The cytotoxic formaldehyde can either be assimilated into cell material or further oxidized to carbon dioxide (26, 27). The assimilation of C_1 in methylotrophic bacteria occurs either via the ribulose monophosphate (RuMP) pathway, the serine cycle or the Calvin-Benson-Bassham cycle. Whereas CO_2 is reduced and converted to biomass in the Calvin-Benson-Bassham cycle, does assimilation of C_1 in the serine cycle occur on the level of methylene-H_4F and CO_2 (28). In the RuMP pathway, formaldehyde and ribulose-5-phosphate are condensed to form D-arabino-3-hexulose-6-phosphate, which can be isomerized to fructose-6-phosphate. These reactions are catalyzed by a 3-hexulose-6-phosphate synthase (HPS) and a 6-phosphate-3-hexuloisomerase (PHI), respectively. Fructose-6-phosphate can be converted to pyruvate via glycolysis or the Entner-Doudoroff pathway, or used to regenerate the formaldehyde acceptor ribulose-5-phosphate via several reactions of the pentose phosphate pathway (29).

In this work, we describe the functional implementation of methanol oxidation and formaldehyde assimilation via the RuMP pathway in *C. glutamicum* strains and present promising approaches to use methanol as auxiliary substrate during growth in sugar-based defined medium

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. *C. glutamicum* was routinely cultivated aerobically in either 500 mL baffled shake flasks with 50 mL medium on a rotary shaker (120 rpm) at 30°C, or in 48-well FlowerPlates (m2p-labs, Aachen, Germany) filled with 750 µL medium in a BioLectorBasic (m2p-labs, Aachen, Germany) at 900 rpm, 30°C and 80% humidity. Growth in shake flasks was monitored by measuring the optical density at 600 nm (OD_{600}) and growth in the BioLectorBasic was monitored online by measuring the backscatter at 620 nm (gain = 12). The gravimetric determination of cell dry weight (CDW) was performed in triplicate by centrifugation of 2 ml culture broth in pre-dried and pre-weighed 2 ml Eppendorf tubes. The pelleted cells were dried for 48 h at 80°C and weighed afterwards. LB medium (30) was used for 5 mL pre-cultures and main-cultures were grown in modified CGXII defined medium (31) containing 55 mM glucose. Methanol was added to the culture medium to a final concentration of 120 mM. For strain construction

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	F^- φ80dlacΔ(*lacZ*)M15 Δ (*lacZYA-argF*) U169 *endA*1 *recA*1 *hsdR*17 (r_K^-, m_K^+) *deoR thi*-1 *phoA supE*44 λ^- *gyrA*96 *relA*1	Invitrogen (Karlsruhe, Germany)
B. subtilis		
168	Wild-type, trpC2, auxotrophic for tryptophan	BGSC*
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild-type strain	(25)
Δ*ald*Δ*adhE*	Derivative of ATCC 13032 with an in-frame deletion of the *ald* (cg3096) and *adhE* (cg0387)genes	(14)
Plasmids		
pEKEx2	Kan^R; *C. glutamicum*/ *E. coli* shuttle vector for regulated gene expression; (P_{tac}, $lacI^Q$, pBL1 $oriV_{C.g.}$, pUC18 $oriV_{E.c.}$)	(26)
pVWEx2	Tet^R; *C. glutamicum*/ *E. coli* shuttle vector for regulated gene expression; (P_{tac}, $lacI^Q$, pCG1 $oriV_{C.g.}$, pUC18 $oriV_{E.c.}$)	(27)
pVWEx2-Bm(*mdh-act*)	Tet^R; pVWEx2 derivative containing Bm(*mdh-act*) under control of P*tac*	This work
pVWEx2-P*tuf*-Bm(*mdh-act*)	Tet^R; pVWEx2 derivative containing Bm(*mdh-act*) under control of P*tuf*	This work
pVWEx2-Bm(*mdh3-act*)	Tet^R; pVWEx2 derivative containing Bm(*mdh3-act*) under control of P*tac*	This work
pVWEx2-P*tuf*-Bm(*mdh3-act*)	Tet^R; pVWEx2 derivative containing Bm(*mdh3-act*) under control of P*tuf*	This work
pVWEx2-Cg*adhA*	Tet^R; pVWEx2 derivative containing Cg*adhA* under control of P*tac*	This work
pEKEx2-Bs(*hps-phi*)	Kan^R; pEKEx2 derivative containing Bs(*hps-phi*) under control of P*tac*	This work
pEKEx2- P*tuf* -Bs(*hps-phi*)	Kan^R; pEKEx2 derivative containing Bs(*hps-phi*) under control of P*tuf*	This work
pEKEx2-Mg(*hps-phi*)	Kan^R; pEKEx2 derivative containing Mg(*hps-phi*) under control of P*tac*	This work

* Bacillus Genetic Stock Center

and maintenance, either LB or BHIS agar plates [BHI agar (Difco,Detroit, MI, USA) supplemented with 0.5 M sorbitol] were used. *Escherichia coli* DH5α was used for cloning purposes and was grown aerobically on a rotary shaker (170 rpm) at 37°C in 5 mL LB medium or on LB agar plates [LB medium with 1.8% (w/v) agar]. If appropriate, kanamycin and/or tetracycline were added to final concentrations of 25 µg mL^{-1} / 5 µg mL^{-1} (*C. glutamicum*) or 50 µg mL^{-1} / 12.5 µg mL^{-1} (*E. coli*).

Strain construction. The enzymes for recombinant DNA work were obtained from Fermentas (St. Leon-Rot, Germany) and Merck Millipore (Billerica, MA, USA). *E. coli* was transformed by the RbCl method (32) and *C. glutamicum* ATCC 13032 by electroporation (33). Routine methods like PCR, restriction or ligation were carried out according to standard protocols (34). The oligonucleotides used for cloning were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1. The genes *mdh*, *mdh3* and *act* originating from *B. methanolicus* MGA3 (ATCC 53907) (NCBI gene accession numbers: EIJ77596, EIJ80770 and AAM98772, respectively) were optimized for expression in *C. glutamicum* by adapting the gene sequence to the codon-usage of *C. glutamicum*. These genes, as well as the wild-type coding sequence of the operon consisting of *rmpA* (from now on designated as *hps*) and *rmpB* (from now on designated as *phi*) from *M. gastri* (NCBI gene accession numbers: Q9LBW4 and Q9LBW5, respectively) were ordered from Life Technologies GmbH (Darmstadt, Germany). All genes, synthesized with desired restriction sites for sub-cloning into *C. glutamicum* expression vectors, were provided as purified DNA on standard vectors of Life Technologies. The *adhA* gene (cg3107) was amplified from genomic DNA of *C. glutamicum* ATCC 13032 by PCR. The genes *yckG* (from now on designated as *hps*) and *yckF* (from now on designated as *phi*) were amplified from genomic DNA of *Bacillus subtilis* 168 by PCR. For the heterologous gene expression under the control of a constitutive promoter, the P*tuf* (promoter of the elongation factor Tu) was first amplified by PCR from genomic DNA of *C. glutamicum* (14 bp – 179 bp upstream of the *tuf* start codon) and subsequently cloned in front of the above mentioned genes. The correct DNA sequences were verified by DNA sequencing using plasmid-specific primers. Detailed cloning procedures of the constructed vectors are described in the supplementary material.

Determination of methanol by gas chromatography. The quantitative measurement of methanol in the cell-free supernatant of *C. glutamicum* cultures was performed by capillary gas chromatography (GC) using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) as described previously (22).

Enzyme assays in crude extracts. Recombinant *C. glutamicum* strains were cultivated in 100 ml CGXII defined medium with 55 mM glucose and 120 mM methanol at 30°C and 120 rpm in shake flasks. Induction of gene expression was performed with 1.5 mM IPTG at an OD_{600} of 1.0. For the determination of enzyme activities in the exponential and in the stationary phase, 50 ml of the cell culture were harvested at an OD_{600} of 5 and 30 ml were harvested after 12 h of cultivation by centrifugation (4500 x *g*, 15 min, 4°C). Cells were washed with 100 mM glycine-KOH buffer, pH 9.4 (Mdh / Mdh3 enzyme assay) or with 50 mM potassium phosphate buffer, pH 7.6 containing 1 mM dithiothreitol and 3 mM $MgCl_2$ (HPS/PHI enzyme assays). The cell pellets from the exponential phase and the stationary phase were resuspended in 500 µl and 800 µl buffer, respectively. For crude cell extract preparation, mechanical lysis of cells was performed with glass beads (diameter: 0.1 mm, 350 mg in a 1.5 ml screw-cap tube) for 3 x 20 s using the Precellys24 (Bertin Technologies, Montigny-le-Bretonneux, France) and centrifuged for 30 min at 4°C and 16.000 x *g* to remove the cell debris. The supernatant was used for the enzyme assays. The enzyme assays were performed in 96-well plates in 200 µl scale at 30°C by following the increase in absorption at 340 nm using the Infinite M200 Pro TECAN (Tecan Group AG, Männedorf, Switzerland). The protein concentration of the cell-free extracts was determined by the method of Bradford (35) using bovine serum albumin as standard.

The methanol dehydrogenase assay was performed as described previously (36) with slight

modifications. The assay mixtures contained 100 mM glycine-KOH buffer, pH 9.4, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM NAD^+ and 40 µl cell-free extract in different dilutions. The reaction was initiated by the addition of 500 mM methanol and monitored over 3 min. 1U of methanol dehydrogenase activity was defined as the reduction of 1 µmol NAD^+ to NADH per minute.

The coupled HPS/PHI assay was performed as described previously (37), but with minor modifications of the protocol. Briefly, determination of HPS/PHI activities through measurement of NADPH formation requires the activity of three additional enzymes in the assay: phosphoriboisomerase (PRI), phosphogluco-isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PDH). The assay mixtures contained 50 mM potassium phosphate buffer, pH 7.6, 5 mM $MgCl_2$, 5 mM ribose-5-phosphate, 2.5 mM $NADP^+$, 5 U PGI from yeast (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), 5 U G6PDH from yeast (grade II, Roche Diagnostics Deutschland GmbH, Mannheim, Germany), 5 U PRI from spinach (Type I, partially purified powder, Sigma-Aldrich, St. Louis, MO, USA) and 40 µl cell-free extract in different dilutions. The reaction was incubated for 5 min at 30°C to ensure equilibrium between ribose-5-phosphate and ribulose-5-phosphate. Subsequently, formaldehyde (37% formaldehyde solution, Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 5 mM to start the reaction. The reaction was monitored for 15 min. 1 U of coupled HPS/ PHI activity was defined as the reduction of 1 µmol $NADP^+$ to NADPH per minute.

Determination of ^{13}C-labeled intracellular metabolites and $^{13}CO_2$. The assimilation of methanol in recombinant *C. glutamicum* strains was monitored in ^{13}C-methanol labeling experiments. Cells were cultivated in 200 ml CGXII defined medium with 55 mM glucose and 120 mM ^{13}C-labeled methanol (99% atom enrichment, Sigma-Aldrich, St. Louis, MO, USA) in a DASGIP Parallel Bioreactor System (DASGIP AG, Jülich, Germany). During the cultivation, $^{13}CO_2$ and $^{12}CO_2$ off-gas analysis was performed as described previously (22). Analysis of the ^{13}C-labeled intracellular metabolites was performed in the exponential growth phase, in the transition from the exponential growth phase to the stationary phase as well as 12h and 24h after reaching the stationary phase. Quenching of the metabolic activity as well as extraction and analysis of intracellular metabolites was performed as previously described (38). Raw mass spectrometry data was corrected for the contribution of all naturally abundant isotopes as well the isotopic impurity of the tracer using the software IsoCor (39).

RESULTS

Design of a heterologous pathway for the assimilation of methanol-derived carbon in *C. glutamicum*. The first step of engineering *C. glutamicum* towards utilization of methanol was the heterologous expression of a methanol dehydrogenase (MDH) to oxidize methanol to formaldehyde (Fig. 1). The focus was put on NAD^+-dependent MDH's since pyrroloquinolin quinone (PQQ)-dependent MDH's are not suitable for an expression in *C. glutamicum* as this organism does not synthesize PQQ or possess any PQQ-dependent enzymes (43). *B. methanolicus* MGA3 is a well-known methylotroph and contains three genes encoding for NAD^+-dependent MDH's (*mdh*, *mdh2* and *mdh3*) and the *act* gene encoding the MDH activator protein (Act), which is important for the activity of all three MDHs (44, 45). In presence of Act and with methanol as substrate, the Mdh and Mdh3 show the highest activity *in vitro* (0.5 U/mg and 0.2 U/mg, respectively), whereas the Mdh2 shows the lowest catalytic activity (0.14 U/mg) (45). Thus, *mdh*, *mdh3* and *act* genes were codon-optimized for an expression in *C. glutamicum* and commercially

synthesized [(Bm(*mdh-act*) and Bm(*mdh3-act*)]. In addition, the AdhA of *C. glutamicum* was previously shown to contribute in endogenous oxidation of methanol to formaldehyde (22). With the aim to simply increase the endogenous methanol oxidation rate, the respective gene (Cg*adhA*) was also chosen as an alternative target for overexpression in *C. glutamicum*.

We decided to pursue the implementation of the ribulose monophosphate (RuMP) pathway for methanol assimilation in *C. glutamicum* as establishing this pathway demands only the functional expression of genes for a 3-hexulose-6-phosphate synthase (HPS) and a 6-phosphate-3-hexuloisomerase (PHI). All other enzymatic activities required for the conversion of fructose-6-phosphate and regeneration of ribulose-5-phosphate as C_1-acceptor can be recruited from the central metabolism of *C. glutamicum* (Fig. 1). Furthermore, these two enzymes require no cofactors and the assimilation of C_1 via the RuMP pathway occurs directly on the level of formaldehyde and not on the level of methylene-H_4F and/or CO_2 as in the serine cycle (28, 29). Hence, we decided to evaluate the functionality of the HPS and the PHI from the non-methylotroph *B. subtilis,* [Bs(*hps-phi*)] and from the methylotroph *Mycobacterium gastri* [Mg(*hps-phi*)] to constitute a functional RuMP pathway in *C. glutamicum*.

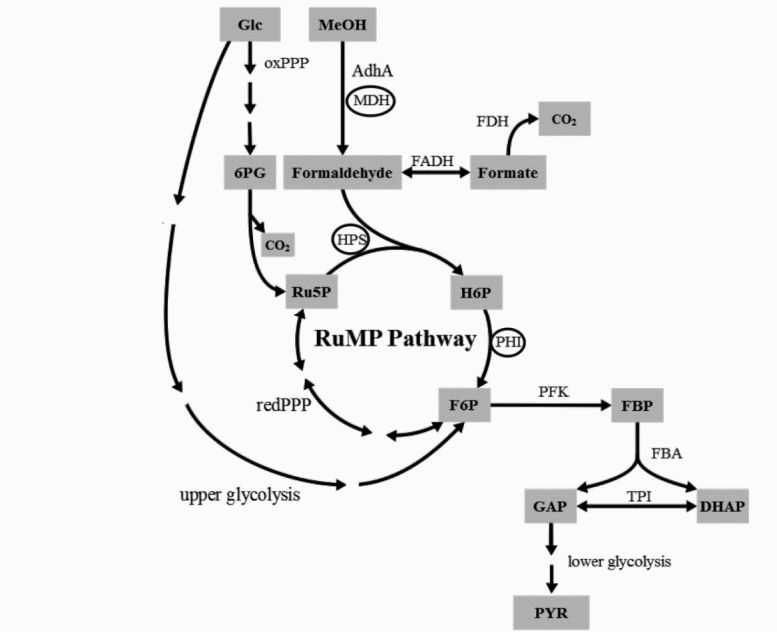

FIG 1 Simplified overview of the methanol and formaldehyde oxidation as well as the formaldehyde assimilation via the ribulose monophosphate (RuMP) pathway in context of the central carbon metabolism of *C. glutamicum*. Abbreviations: oxPPP/redPPP, oxidative/reductive pentose phosphate pathway; MDH, methanol dehydrogenase; AdhA, alcohol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phosphate-3-hexuloisomerase; PFK, phosphofructokinase; FBA, fructose bisphosphate aldolase; TPI, triosephosphate isomerase; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; H6P, hexulose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PYR, pyruvate. The three enzymes [MDH, HPS and PHI] necessary for establishing a synthetic pathway for methanol oxidation and formaldehyde assimilation in *C. glutamicum* are encircled.

Implementation of a synthetic pathway for methanol oxidation and formaldehyde assimilation in *C. glutamicum* wild type. For growth on methanol, methylotrophic organisms require a pathway for the assimilation of formaldehyde to generate biomass as well as a pathway for the dissimilation of formaldehyde to generate energy. The dissimilatory pathway also serves as "safety valve" in case of accumulation of formaldehyde in toxic amounts. Since *C. glutamicum* wild type already possesses an endogenous pathway for the oxidation of naturally occurring cytotoxic formaldehyde to CO_2 (22, 23), we decided to initially express the genes of the synthetic methanol assimilation pathway in this strain. Two plasmids with the IPTG-inducible promoter P*tac* were used for the heterologous gene expression: one for the expression of the modules for methanol oxidation [Cg*adhA* or Bm(*mdh-act*)] and one for the expression of the modules for formaldehyde assimilation [Bs(*hps-phi*) or Mg(*hps-phi*)]. Thus, Cg*adhA* or Bm(*mdh-act*) were individually expressed in combination with Bs(*hps-phi*) or Mg(*hps-phi*) in *C. glutamicum*.

Initially, the modules for formaldehyde assimilation were evaluated via conducting *in vitro* enzyme assays with crude extracts of recombinant *C. glutamicum* strains. These revealed that only the assimilation module Bs(Hps-Phi) is functionally active in *C. glutamicum* (73 ± 25 mU/mg in the exponential growth phase), whereas negligible activity (1.4 ± 2 mU/mg) could be detected for Mg(Hps-Phi) (Table 2A). Consequently, strains containing Mg(*hps-phi*) were not further characterized. The functionality of the modules for methanol oxidation was tested by measuring the methanol oxidation rate of recombinant *C. glutamicum* strains during shake flask cultivations in medium containing 55 mM glucose and 120 mM methanol. The strain expressing Cg*adhA* and Bs(*hps-phi*) consumed methanol only slightly faster compared to the control strain (*C. glutamicum* pVWEx2 pEKEx2) (data not shown). In contrast, the methanol oxidation rate of the recombinant *C. glutamicum* strain expressing Bm(*mdh-act*) and Bs(*hps-phi*) was nearly 3-fold higher (1.3 ± 0.2 mM/h) than the one of the control strain (0.5 mM/h ± 0.1) (Fig. 2A). Due to these results, we excluded the strain expressing Cg(*adhA*) from further experiments and put our focus on *C. glutamicum* strain expressing Bm(*mdh-act*) and Bs(*hps-phi*).

Table 2 Specific activities of Bm(Mdh-Act) and Bm(Mdh3-Act) and the coupled specific activities of Bs(Hps-Phi) as well as of Mg(Hps-Phi) in crude cell extracts of (**A**) recombinant *C. glutamicum* strains and (**B**) recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains. Cells were cultivated in CGXII defined medium with 55 mM glucose and 120 mM methanol at 30°C by shaking at 120 rpm. Genes under control of P*tac* were induced with 1.5 mM IPTG at an OD_{600} of 1.0. Mean values and standard deviations were calculated from triplicates and values were corrected for background activity. The background activity was determined by enzyme assays with the crude cell extracts of *C. glutamicum* (pVWEx2 pEKEx2) and *C. glutamicum* Δ*ald*Δ*adhE* (pVWEx2 pEKEx2). n.d.: not determined

Strain	**Coupled HPS-PHI activity [mU/mg]**		**Mdh/Mdh3 activity [mU/mg]**	
	Exponential phase	**Stationary phase**	**Exponential phase**	**Stationary phase**
A *C. glutamicum*				
Bm(*mdh-act*) Bs(*hps-phi*)	73.3 ± 24.8	22.1 ± 7.9	0.4 ± 0.2	0.7 ± 0.5
Bm(*mdh-act*) Mg(*hps-phi*)	1.4 ± 1.6	1.0 ± 1.4	n.d.	n.d.
P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*)	53.1 ± 23.5	13.7 ± 1.3	3.3 ± 1.2	2.0 ± 1.5
B *C. glutamicum* Δ*ald*Δ*adhE*				
P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*)	45.3 ± 9.7	24.3 ± 15.3	5.5 ± 1.1	2.2 ± 1.8
P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*)	36.3 ± 4.9	22.3 ± 12.1	6.0 ± 1.5	5.2 ± 1.9
P*tuf*-Bm(*mdh-act*) pEKEx2	n.d.	n.d.	5.5 ± 1.1	3.2 ± 2.3

Enzyme assays conducted with crude extract samples taken at different time points during cultivation revealed negligible MDH-activity in the exponential growth phase; in the stationary phase a slightly higher activity was measurable (0.7 ± 0.5 mU/mg; Table 2A). These results were in line with the observations that methanol oxidation was more significant in the stationary phase (Fig. 2A). Since IPTG-inducible expression of Bm(*mdh-act*) seems not to be suitable due to the negligible activity of Bm(*mdh-act*) in the exponential growth phase, we also evaluated the application of a strong constitutive promoter to control heterologous gene expression. For this purpose, the expression of both modules [methanol oxidation (Bm(*mdh-act*) and formaldehyde assimilation (Bs(*hps-phi*)] was set under the control of the constitutive promoter P*tuf*. Enzyme assays with crude cell extract of the respective strain revealed that the specific MDH-activity in the exponential growth phase was significantly increased from 0.4 ± 0.2 mU/mg to 3.3 ± 1.2 mU/mg (Table 2A). During growth of *C. glutamicum* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) in glucose and methanol containing medium, methanol oxidation started in the exponential growth phase and a higher average methanol consumption rate (1.7 ± 0.3 mM/h) compared to the strain expressing both modules under control of the inducible promoter P*tac* (1.3 ± 0.2 mM/h) was obtained (Fig. 2A).

Initial growth experiments in microtiter plates showed that all recombinant strains and the control strain *C. glutamicum* pVWEx2 pEKEx2 grew similar in medium containing 55 mM glucose without methanol (Fig. 2B). In the presence of 120 mM methanol, severe differences in growth and final backscatter of the cultures could be observed (Fig. 2C). The growth of the control strain and of the recombinant strain expressing the methanol oxidation and formaldehyde assimilation modules under control of P*tac* was significantly retarded, revealing an inhibitory effect of methanol. The latter strain could cope slightly better with the presence of methanol, as it grew faster during the exponential growth phase. However, this growth advantage cannot be explained with the observed higher methanol oxidation of the recombinant strain (1.3 ± 0.2 mM/h) in comparison to the control strain (0.5 ± 0.1 mM/h) during the course of the cultivation since both strains hardly consumed methanol in the exponential phase (Fig. 2A). At the beginning of the stationary phase, backscatter measurements showed a drop of the cell density in both cultures (Fig. 2C). In contrast to the control strain, *C. glutamicum* Bm(*mdh-act*) Bs(*hps-phi*) cells seem to recover during the course of the stationary phase, leading to a 1.3-fold higher final backscatter of this culture (Fig. 2C) and an increased cell dry weight (CDW) by 4% (Table 3A) in comparison to the control strain. The same was observed for a *C. glutamicum* strain that exclusively expressed the methanol oxidation module but not the formaldehyde assimilation module (Fig. S1). This leads to the assumption that the higher final backscatter of the C. *glutamicum* Bm(*mdh-act*) Bs(*hps-phi*) culture compared to the control strain *C. glutamicum* pVWEx2 pEKEx2 does not reflect assimilation of methanol-derived carbon, but could only be due to a lower methanol concentration in the medium during the stationary phase, diminishing its inhibitory effect.

In contrast, the *C. glutamicum* strain expressing the genes for methanol oxidation and formaldehyde assimilation under control of P*tuf* showed a similar growth in defined medium with and without methanol. In comparison to the *C. glutamicum* strain expressing same genes under control of P*tac*, a higher backscatter of the culture at the end of exponential growth was detected. Moreover, an additional increase of the cell density during the "stationary phase" in methanol containing medium was observed, leading to a significantly higher final backscatter and to an 18% increased CDW (Table 3A). Remarkably, it also grew to a higher final backscatter in the presence of methanol (Fig. 2C) and also showed a slightly increased CDW under these conditions (8.4 ± 0.03 vs. 8.0 ± 0.05 mg/ml). This hints towards the ability of this *C. glutamicum* strain to convert methanol-derived carbon into biomass.

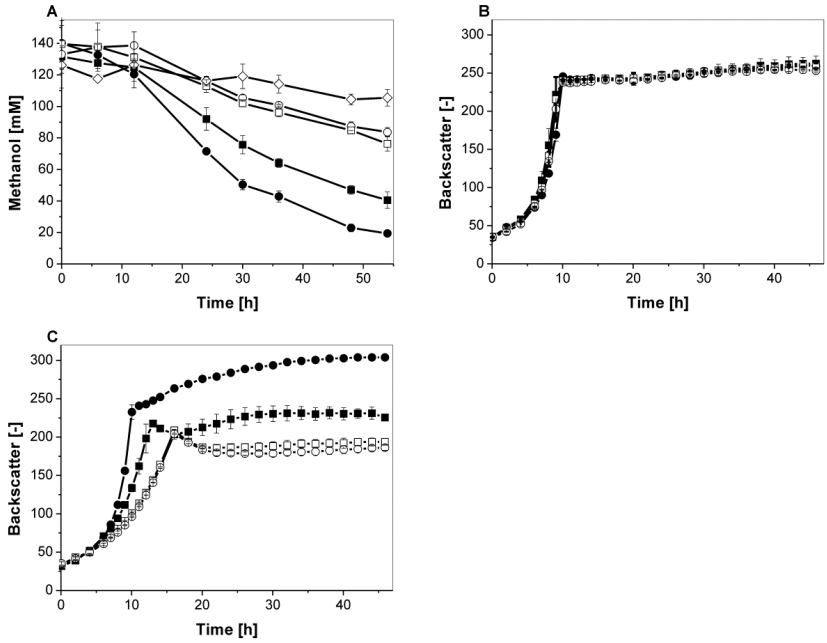

FIG 2 (**A**) Methanol consumption in CGXII defined medium supplemented with 55 mM glucose and 120 mM methanol. (**B**) Growth in CGXII defined medium supplemented with 55 mM glucose. (**C**) Growth in CGXII defined medium supplemented with 55 mM glucose and 120 mM methanol of recombinant *C. glutamicum* strains. Induction of gene expression was performed with 1.5 mM IPTG when genes are expressed under control of the P*tac*. For determination of the methanol concentration, three independent experiments in shake flasks, always accompanied by a methanol evaporation control, were performed (◊). Monitoring of growth was performed in 48-well FlowerPlates using a BioLectorBasic (n=3). Strains: *C. glutamicum* Bm(*mdh-act*) Bs(*hps-phi*) (■), *C. glutamicum* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) (●), *C. glutamicum* pVWEx2 pEKEx2 (+ IPTG) (□) and *C. glutamicum* pVWEx2 pEKEx2 (- IPTG) (○).

Table 3 Cell dry weight of (**A**) recombinant *C. glutamicum* strains and (**B**) recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains in glucose (55 mM) and methanol (120 mM) containing CGXII defined medium. The CDW was determined 32 h after inoculation (stationary phase). Genes under control of P*tac* were induced with 1.5 mM IPTG at an OD_{600} of 1.0. Mean values and standard deviations were calculated from triplicates.

Strain	**CDW [mg/ml culture broth]**
A ***C. glutamicum***	
Bm(*mdh-act*) Bs(*hps-phi*)	6.72 ± 0.05
P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*)	8.37 ± 0.03
control (pVWEx2 pEKEx2)	6.49 ± 0.13
B ***C. glutamicum* Δ*ald*Δ*adhE***	
P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*)	6.75 ± 0.01
P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*)	7.14 ± 0.13
P*tuf*-Bm(*mdh-act*) pEKEx2	4.87 ± 0.25
control (pVWEx2 pEKEx2)	6.35 ± 0.10

Methanol-derived carbon is assimilated into intracellular metabolites of *C. glutamicum*. ^{13}C-methanol labeling experiments were performed to validate that expression of P*tuf*-Bm(*mdh-act*) and P*tuf*-Bs(*hps-phi*) results in the incorporation of methanol-derived carbon into intracellular metabolites of the respective strain. Two independent batch fermentations were run in CGXII medium supplemented with 55 mM glucose and 120 mM ^{13}C-methanol. Quenching of the metabolic activity as well as analysis of the intracellular metabolites was performed in the exponential growth phase, at the point of transition to the stationary phase as well as 12 h and 24 h after reaching the stationary phase. In the exponential growth phase, incorporation of ^{13}C-methanol-derived carbo into intracellular metabolites could already be detected, although amounts were quite low (up to 5% labeling fractions in the m+1 mass isotopomers) (Fig. S2). With the entrance to the stationary phase, labeling fractions of 3-10% in the m+1 mass isotopomers of various metabolites such as the organic acids succinate and α-ketoglutarate, the sugar phosphates glucose-6-phosphate and fructose-1,6-bisphosphate as well as the amino acids L-serine and L-lysine, were detected (Table 4). For some metabolites, the labeling fractions in the m+1 mass isotopomers was even increased after further 12 h of cultivation (Fig. S4). No ^{13}C-labeling that was higher than the natural ^{13}C-abundance could be detected in any metabolites of the negative control *C. glutamicum* pVWEx2 pEKEx2 (data not shown). In comparison to this control, a high $^{13}CO_2$/total CO_2

Table 4 Incorporation of ^{13}C-methanol-derived carbon into intracellular metabolites of *C. glutamicum* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*). For each strain, two batch reactors (BR1, BR2) were run with CGXII defined medium supplemented with 55 mM glucose and 120 mM ^{13}C-labeled methanol. Quenching of metabolic activity as well as intracellular metabolic analysis was performed in the transition to the stationary phase. Mass isotopomer measurements were performed for selected metabolites and the corresponding m+1 mass traces were listed. Raw mass spectrometry data was corrected for the contribution of all naturally abundant isotopes. n.d.: not determined.

Metabolite	**m+1 labeled [%]**	
	BR1	**BR2**
Glucose-6-phosphate	2.7	3.6
6-phosphogluconate	2.0	n.d.
Sedoheptulose-7-phosphate	6.0	6.2
Fructose-1,6-bisphosphate	8.2	6.2
Dihydroxyacetone phosphate	3.2	3.0
2-/3- phosphoglycerate	4.0	5.0
Phosphoenolpyruvate	1.9	4.2
Pyruvate	5.6	5.1
α-Ketoglutarate	8.0	8.3
Succinate	8.5	8.7
Histidine	n.d.	6.9
Serine	6.1	8.5
Homoserine	4.8	4.5
Tryptophan	10.7	9.9
Tyrosine	10.1	9.2
Phenylalanine	7.8	7.8
Valine	6.8	5.7
Alanine	7.0	7.1
Proline	5.6	5.2
Arginine	4.5	3.6
Glutamine	6.0	6.2
Lysine	8.3	7.7
Aspartate	9.3	9.1
Threonine	5.1	6.4

ratio for the strain expressing the methanol oxidation and formaldehyde assimilation modules under control of P*tuf* could be observed (Fig. 3). This led us to the assumption that most of the ^{13}C-methanol-derived formaldehyde is oxidized to $^{13}CO_2$ via the endogenous pathway for formaldehyde dissimilation.

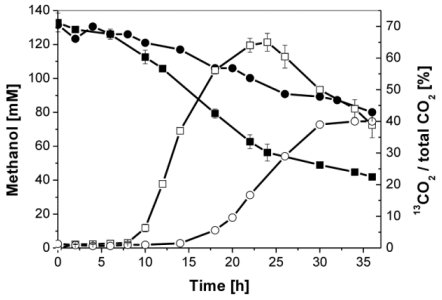

FIG 3 Methanol consumption (filled symbols) and $^{13}CO_2$ formation as $^{13}CO_2$/total CO_2 ratio (open symbols) of *C. glutamicum* expressing P*tuf*-Bm(*mdh-act*) and P*tuf*-Bs(*hps-phi*) (■) and the negative control *C. glutamicum* pVWEx2 pEKEx2 (●) during cultivation in bioreactors in CGXII defined medium supplemented with 55 mM glucose and 120 mM ^{13}C-labeled methanol. Two independent experiments were performed and mean values are shown.

Implementation of methanol oxidation and formaldehyde assimilation in *C. glutamicum* Δ*ald*Δ*adhE*. ^{13}C-methanol labeling experiments revealed that only a low amount of ^{13}C-methanol-derived carbon was assimilated into biomass precursors of a *C. glutamicum* strain expressing genes for methanol oxidation and formaldehyde assimilation (Table 4). Consequently, P*tuf*-Bm(*mdh-act*) and P*tuf*-Bs(*hps-phi*) as well as only P*tuf*-Bm(*mdh-act*) were expressed in *C. glutamicum* Δ*ald*Δ*adhE*, a strain which is strongly impaired in its ability to oxidize formaldehyde to CO_2 (22, 23). This approach should prevent loss of methanol-derived formaldehyde as CO_2 and increase formaldehyde assimilation. In addition, *C. glutamicum* Δ*ald*Δ*adhE* pVWEx2 pEKEx2, only harbouring the empty plasmids, was constructed as control. All strains were analysed regarding their growth and methanol consumption rate to determine the impact of the absence of the "safety valve" for formaldehyde oxidation during growth in the presence of methanol.

Determination of the *in vitro* enzyme activities revealed that all enzymes were functionally active in recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains (Table 2B). In glucose containing medium, growth of all strains was similar (Fig. 4B) and comparable to the strains based on the *C. glutamicum* wild type (Fig. 2B). In comparison, the control strain *C. glutamicum* Δ*ald*Δ*adhE* pVWEx2 pEKEx2 showed a retarded growth and a 33% reduced final backscatter in the presence of methanol. The *C. glutamicum* Δ*ald*Δ*adhE* strain only expressing P*tuf*-Bm(*mdh-act*) showed an even slower growth in the presence of methanol and the final backscatter of the culture was reduced by additional 35% (Fig. 4C). The methanol consumption rate was as low as observed for the control strain (0.52 ± 0.1 mM/h) (Fig. 4A). This strain possesses the heterologous methanol dehydrogenase for oxidation of methanol to formaldehyde, but due to the absence of Bs(Hps-Phi) and the lack of Ald and AdhE, toxic accumulation of formaldehyde cannot be avoided, neither via assimilation by the RuMP pathway, nor via dissimilation to CO_2.

The *C. glutamicum* Δ*ald*Δ*adhE* strain expressing both, the module for methanol oxidation [P*tuf*-Bm(*mdh-act*)] as well as the module for formaldehyde assimilation [P*tuf*-Bs(*hps-phi*)] showed an increased methanol oxidation rate (1.25 ± 0.2 mM/h) in comparison to the control strain and methanol oxidation started in the exponential growth phase. In addition, this recombinant *C. glutamicum* Δ*ald*Δ*adhE* strain had a growth advantage over the control strain in the presence of methanol, since the backscatter on this culture was found to be 15% higher at the end of the exponential growth phase, which also resulted in a higher final CDW (6.75 ± 0.00 mg/mL and 4.87 ± 0.25 mg/mL, respectively). Differently to the *C. glutamicum* wild type strain expressing P*tuf*-

Bm(*mdh-act*) and P*tuf*-Bs(*hps-phi*), the recombinant *C. glutamicum* Δ*ald*Δ*adhE* strain did not grew in methanol containing medium as fast as in medium without methanol and the final backscatter of the culture was not increased in the presence of methanol in comparison to growth without methanol.

Finally, we also evaluated Mdh3, the other MDH of *B. methanolicus*, in the *C. glutamicum* Δ*ald*Δ*adhE* strain background. P*tuf*-controlled expression of *mdh3* and *act* in combination with P*tuf*-Bs(*hps-phi*) and cultivation in glucose and methanol containing medium revealed a methanol consumption, which was similar to the one of *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) (Fig. 4A). However, with this strain a slightly higher final backscatter of the culture and an increased CDW was reached (Fig. 4B, Table 3B).

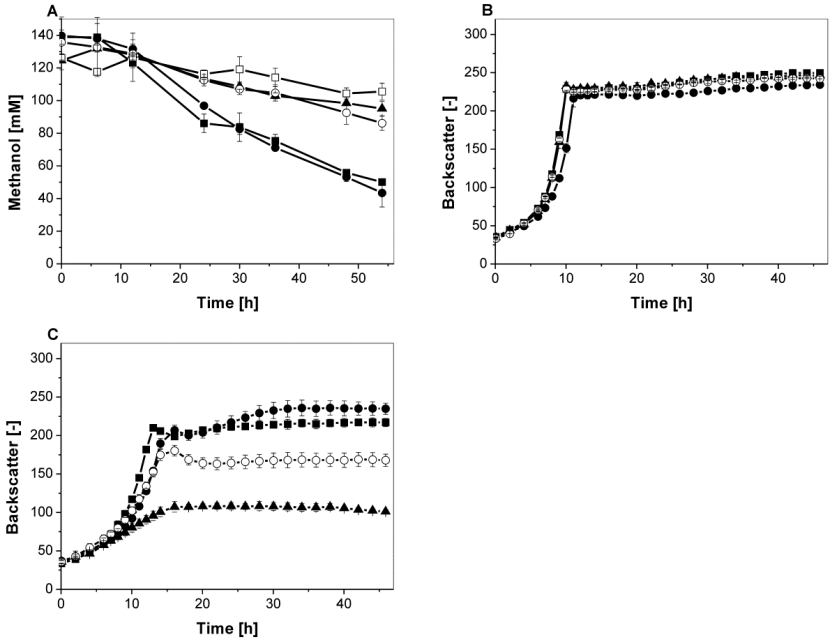

FIG 4 (**A**) Methanol consumption in CGXII defined medium supplemented with 55 mM glucose and 120 mM methanol. (**B**) Growth in CGXII defined medium supplemented with 55 mM glucose. (**C**) Growth in CGXII defined medium supplemented with 55 mM glucose and 120 mM methanol of recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains. For determination of the methanol concentration, three independent experiments in shake flasks, always accompanied by a methanol evaporation control, were performed (◊). Monitoring of growth was performed in 48-well FlowerPlates using a BioLectorBasic (n=3). Strains: *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) (■), *C. glutamicum* P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*) (●), *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh-act*) pEKEx2 (▲) and *C. glutamicum* Δ*ald*Δ*adhE* pVWEx2 pEKEx2 (○).

Increased assimilation of methanol-derived carbon by *C. glutamicum* Δ*ald*Δ*adhE*. ^{13}C-methanol labeling experiments were performed to answer the question if the elimination of the endogenous pathway for formaldehyde dissimilation results in an increased assimilation of methanol-derived carbon into the biomass, although the determined overall biomass yield was not higher in

the presence of methanol. In addition, methanol consumption and generation of $^{13}CO_2$ was measured (Fig. S3A, S3B). Already in the exponential growth phase, incorporation of ^{13}C-methanol-derived carbon into intracellular metabolites of *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) and of *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*) could be observed. Labeling fractions of up to 11% in the m+1 mass isotopomers were detected. With the entrance to the stationary phase, the labeling fractions were found to be about three-fold higher compared to the *C. glutamicum* wild type strain expressing the methanol oxidation and formaldehyde assimilation modules (Table 4, Fig. 5). For the *C. glutamicum* Δ*ald*Δ*adhE* strain expressing P*tuf*-Bm(*mdh3-act*) and P*tuf*-Bs(*hps-phi*), slightly higher labeling fractions in the m+1 mass isotopomers of intracellular metabolites were observed (Fig. S4, S5).

DISCUSSION

Only few methylotrophic organisms, such as *B. methanolicus* and *M. extorquens*, have been engineered to produce biotechnological interesting compounds from methanol (46-48). In this study, we followed the opposite strategy by engineering the non-methylotrophic platform organism *C. glutamicum* to utilize methanol instead of turning a methylotrophic organism into a production strain.

The *C. glutamicum* wild type is already able to oxidize methanol, predominantly catalysed by the alcohol dehydrogenase AdhA (22). Nonetheless, we evaluated the implementation of the NAD^+-dependent methanol dehydrogenase from *B. methanolicus* for increasing the methanol oxidation rate. Since the *C. glutamicum* wild type already possesses an endogenous pathway for the detoxification of naturally occurring cytotoxic formaldehyde to CO_2 (22, 23), only a pathway for assimilation of formaldehyde into biomass was required. Heterologous expression of genes for a 3-hexulose-6-phosphate synthase (HPS) and a 6-phospho-3-hexuloisomerase (PHI) from methylotrophic organisms, both key enzymes of the ribulose monophosphate (RuMP) pathway, has already been done to confer the capability of formaldehyde assimilation to *Burkholderia cepacia* and *Pseudomonas putida*, respectively (20, 49, 50). However, heterologous expression of *hps* and *phi* from the methylotroph bacterium *M. gastri*, which is closely related to *C. glutamicum,* did not result in active enzymes. As an alternative we tried the expression of *hps* and *phi* of *B. subtilis*, which requires these genes for formaldehyde detoxification (49, 51) and were able to complete a functional RuMP-pathway in *C. glutamicum*.

Functional expression of enzymes for the methanol oxidation [Bm(Mdh-Act)] and formaldehyde assimilation [Bs(Hps-Phi)] in the *C. glutamicum* wild type as well as the selection of a suitable promoter for gene expression (P*tuf*), resulted in a three-fold increased methanol oxidation rate (1.7± 0.2 mM/h) in comparison to the *C. glutamicum* pVWEx2 pEKEx2 control strain (0.5 ± 0.1 mM/h). Furthermore, the inhibitory effect of methanol on growth and biomass formation of this control strain could be compensated by *C. glutamicum* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*). This strain showed the same growth in medium with and without methanol and at the end of the exponential growth phase a higher backscatter compared to the control strain was observed. This effect could be explained by the generation of additional NADH due to methanol dehydrogenase catalysed oxidation of methanol in the exponential growth phase and subsequent generation of ATP via the oxidative phosphorylation. Thus, less glucose has to be dissimilated for energy generation but can be used for synthesis of biomass precursors. This effect on biomass formation was also observed during co-utilization of the C_1 compounds formate and formaldehyde with glucose by *Candida utilis* and *Penicillium chrysogenum* (52, 53) as well as by a *Saccharomyces cerevisiae* strain expressing a

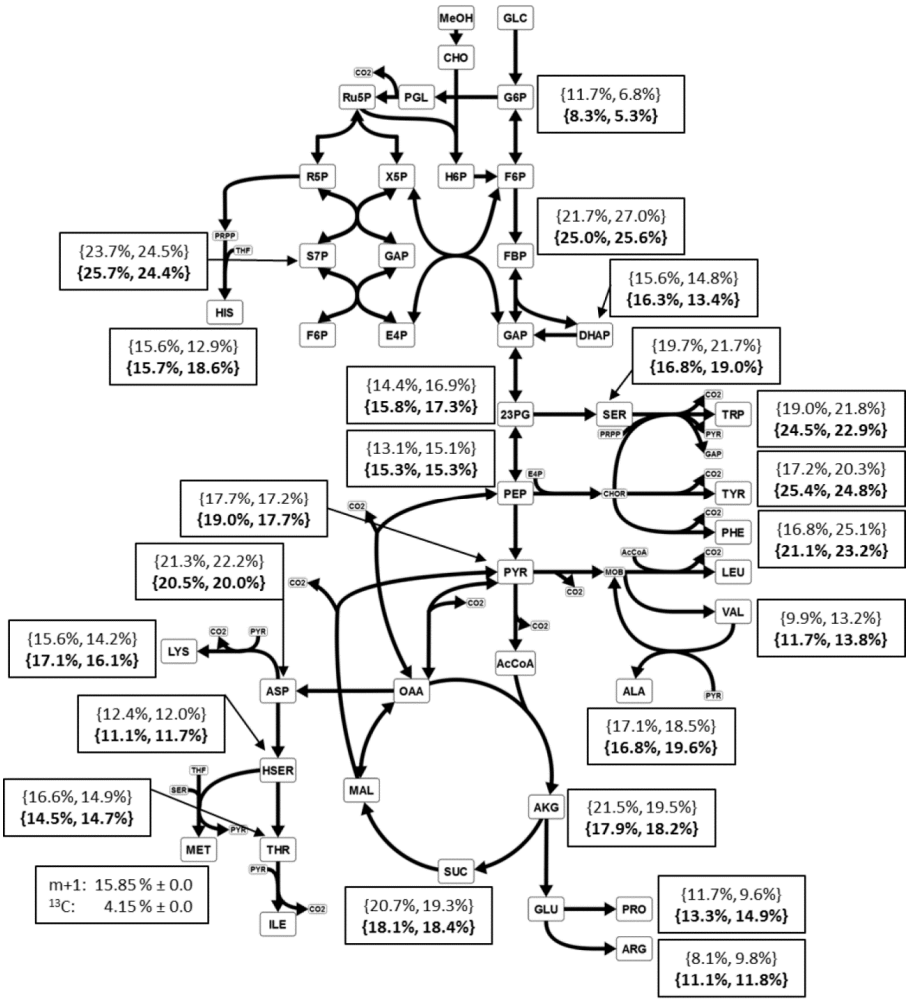

FIG 5 Simplified overview of the central carbon metabolism including methanol oxidation via Bm(Mdh-Act) or Bm(Mdh3-Act) and formaldehyde assimilation via the ribulose monophosphate (RuMP) pathway of recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains. Additionally, the labeling fractions of the m+1 mass isotopomers of the measured intracellular metabolites were listed for the strain *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*) (values in the upper row) and *C. glutamicum* Δ*ald*Δ*adhE* P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*) (values in the lower row, bold)**.** For each strain, two independent batch reactors {BR1, BR2} with CGXII defined medium (55 mM glucose/120 mM ^{13}C-labeled methanol) were run. Quenching of metabolic activity as well as intracellular metabolic analysis was performed in the transition to the stationary phase and raw mass spectrometry data was corrected for the contribution of all naturally abundant isotopes. Abbreviations: amino acids are presented according to their 3-letter standard abbreviations, MeOH, methanol; GLC, glucose; CHO, formaldehyde; G6P, glucose-6-phosphate; PGL, phosphogluconolactone; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrosee-4-phosphate; H6P, hexulose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 23PG, 2-/3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl coenzyme A; AKG, α-ketoglutarate; SUC, succinate; MAL, malate; OAA, oxaloacetate; THF, tetrahydrofuran; PRPP, phosphoribosyl pyrophosphate; CHOR, chorismate.

formaldehyde dehydrogenase and a formate dehydrogenase from *Hansenula polymorpha* (54). Furthermore, the increased biomass formation in recombinant *C. glutamicum* strains could also be explained by assimilation of methanol-derived formaldehyde via HPS and PHI catalyzed reactions. Indeed, ^{13}C-methanol labeling experiments revealed up to 5% labeling fractions in the m+1 mass isotopomers of various intracellular metabolites in samples taken from the exponential growth phase.

After exponential growth in glucose/methanol medium, recombinant *C. glutamicum* cells seem to enter a second growth phase, represented by a slightly increasing backscatter and an increased CDW during the "stationary phase" (8.4 ± 0.03 mg/ml vs. 8.0 ± 0.05 mg/ml in medium without methanol). Again, ^{13}C-methanol labeling experiment showed 3-10% m+1 labeling of intracellular metabolites, indicating assimilation of methanol-derived carbon into biomass. However, the lion's share of methanol was still oxidized to CO_2 via the endogenous pathway for formaldehyde oxidation.

Since implementation of the modules for methanol oxidation and formaldehyde assimilation in *C. glutamicum* wild type resulted in a high flux towards CO_2-formation, we also engineered *C. glutamicum* Δ*ald*Δ*adhE*, a strain severely impaired in its ability to detoxify formaldehyde, for assimilation of methanol. As observed for recombinant *C. glutamicum* wild type strains, the implementation of the modules for methanol oxidation [P*tuf*-Bm(*mdh-act*)] or [P*tuf*-Bm(*mdh3-act*)] and formaldehyde assimilation [P*tuf*-Bs(*hps-phi*)] in *C. glutamicum* Δ*ald*Δ*adhE* resulted in a 15% higher backscatter at the end of the exponential growth phase compared to the control strain *C. glutamicum* Δ*ald*Δ*adhE* pVWEx2 pEKEx2 during growth in methanol containing medium. As discussed before, this effect might be explained with the generation of additional NADH due to methanol oxidation. However, differently to recombinant *C. glutamicum* wild type strains, the oxidation of methanol in recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains comes along with channeling of the methanol-derived formaldehyde into the synthetic RuMP cycle for biomass generation, since *C. glutamicum* Δ*ald*Δ*adhE* is missing the "safety valve" for detoxification of formaldehyde via oxidation to CO_2. Without the assimilation of formaldehyde, toxic amounts of this intermediate would accumulate and inhibit cell growth. Previously, we could show that already 4 mM formaldehyde completely inhibit cell growth of *C. glutamicum* (22). Indeed, ^{13}C-methanol labeling experiments revealed that the m+1 labeling of intracellular metabolites in the exponential growth phase was doubled in comparison to the *C. glutamicum* wild type strain expressing the same genes; in the transition to the stationary phase it was even three-fold higher. However, the elimination of the "safety valve" also led to a reduced methanol consumption rate, a retarded growth and a lowered final CDW, most probably due to accumulation of formaldehyde. Possibly, synthetic formaldehyde assimilation is currently not efficient enough to keep the intracellular formaldehyde concentrations below the toxicity threshold.

In summary, for the first time *C. glutamicum* was engineered to utilize methanol as carbon and energy source during growth in sugar-based defined medium. Furthermore, detection of ^{13}C-labeled amino acids indicates that methanol can also be used as auxiliary substrate for the production of these value-added compounds. For a more efficient use of methanol during sugar-based fermentation processes, methanol oxidation to formaldehyde has to be increased, whereas accumulation of toxic formaldehyde has to be minimized. Thus, balancing of the synthetic formaldehyde assimilation and the endogenous dissimilation has to be achieved. This could be done by metabolic engineering or by employing process engineering strategies, e.g. by developing a fed-batch process with continuous methanol feeding, leading to constant methanol availability during the cultivation as it has been realized for *B. methanolicus* MGA3 (21, 55).

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4 Discussion

Some methylotrophic organisms were already engineered to produce biotechnologically interesting compounds, such as polyhydroxyalkanoates and the amino acids L-glutamate and L-lysine (Brautaset *et al.*, 2010; Mokhtari-Hosseini *et al.*, 2009; Orita *et al.*, 2014). However, the methanol-based amino acid production is not yet economically feasible, although the concept of using methanol as substrate for microbial growth and production is interesting, because the theoretical yield of L-lysine from methanol by *B. methanolicus* is similar (0.71 g L-lysine/ g methanol) to the yield from glucose by *C. glutamicum* (0.68 g L-lysine /g glucose) (Brautaset *et al.*, 2007).

Since the relevance of methanol as feedstock in biotechnological processes is expected to increase in the coming years, the expansion of the substrate spectrum of *C. glutamicum* towards the utilization of methanol as auxiliary substrate was the major aim of this dissertation. For growth on methanol, methylotrophic organism require a pathway for oxidation of methanol, a pathway for assimilation of formaldehyde to generate biomass as well as a pathway for dissimilation of formaldehyde to generate energy and to prevent accumulation of formaldehyde. Initial growth experiments in glucose/methanol defined medium revealed that *C. glutamicum* is able to oxidize methanol in the stationary phase via formaldehyde and formate to CO_2 (0.83 ± 0.2 mM/h, 2.8 ± 0.5 nmol min^{-1} mg CDW^{-1}). Characterization of this pathway detected that the reactions are catalyzed by the alcohol dehydrogenase AdhA, the aldehyde dehydrogenases Ald and AdhE as well as the formate dehydrogenase FdhF. However, *C. glutamicum* is a non-methylotrophic organism and is not able to utilize C_1 compounds for biomass generation. Consequently, the ribulose monophosphate pathway from *B. subtilis* was established in *C. glutamicum* wild type catalyzing the assimilation of formaldehyde [Bs(Hps-Phi)] and a heterologous methanol dehydrogenase from *B. methanolicus* [Bm(Mdh-Act)] was implemented to increase its methanol oxidation rate (FIG 7A). At the same time, this synthetic pathway was implemented into *C. glutamicum* Δ*ald*Δ*adhE*, that is severely impaired in its ability to oxidize formaldehyde, to study the impact of the lack of the dissimilatory pathway on growth, methanol utilization and formaldehyde assimilation (FIG 7B).

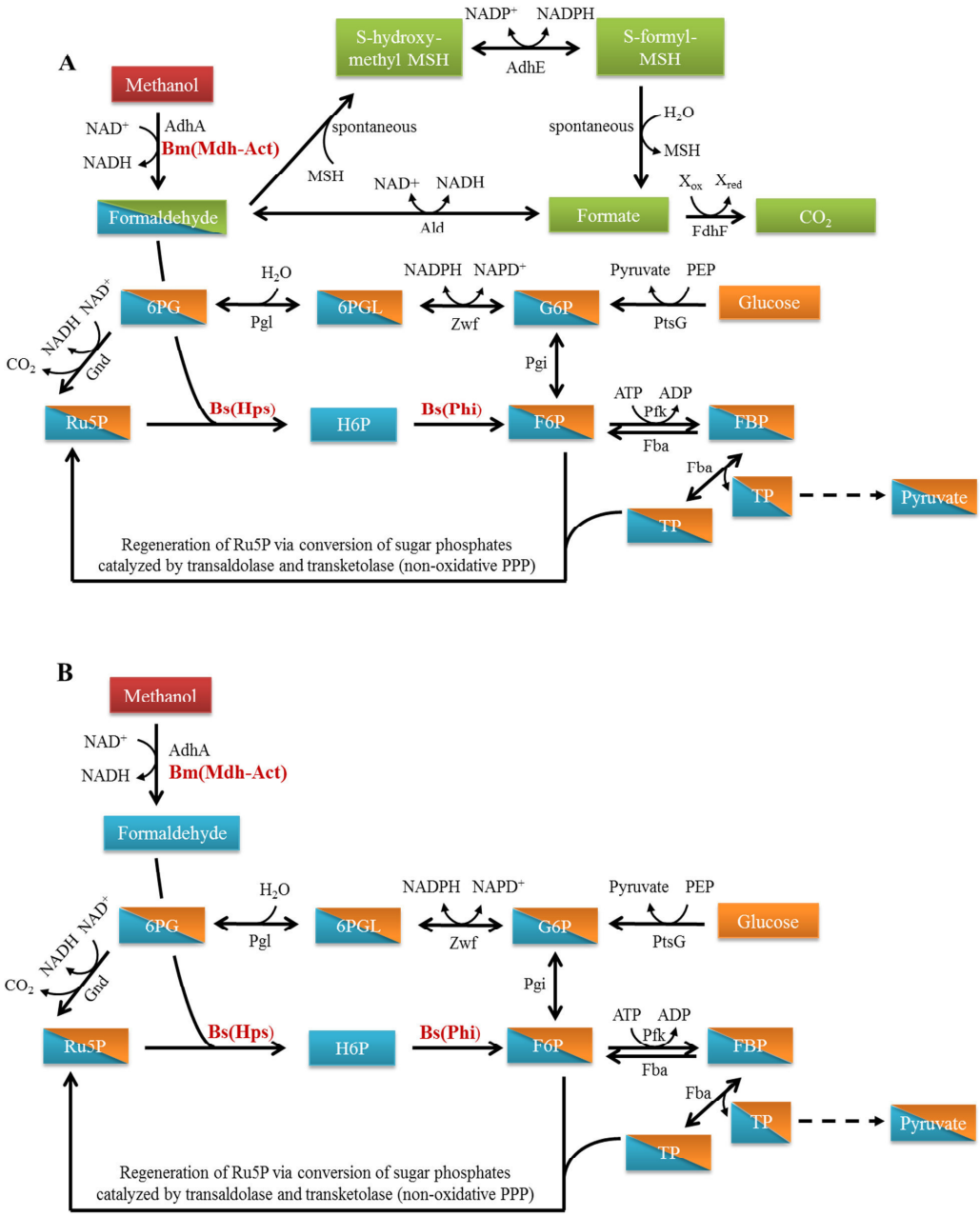

FIG 7 Schematic representations of the metabolization of glucose and methanol in *C. glutamicum* wild type (A) and *C. glutamicum* Δ*ald*Δ*adhE* (B) both harboring heterologous pathways for methanol oxidation and formaldehyde assimilation.

FIG 7 Methanol oxidation (**red**) is catalyzed by the native alcohol dehydrogenase AdhA and by the heterologous methanol dehydrogenases Mdh from *B. methanolicus* [Bm(Mdh-Act)]. In the *C. glutamicum* wild type background (**A**), formaldehyde can either be oxidized to CO_2 (**green**) or assimilated into the central metabolism (**blue**). The oxidation of formaldehyde is catalyzed by the NAD^+-dependent aldehyde dehydrogenase Ald and by the mycothiol (MSH)-dependent alcohol dehydrogenase AdhE. The subsequent oxidation of formate to CO_2 is catalyzed by the formate dehydrogenase FdhF. In the *C. glutamicum* Δ*ald*Δ*adhE* background (**B**), formaldehyde can only be assimilated (**blue**) since the pathway for formaldehyde oxidation via Ald and AdhE was deleted. In both strains, assimilation of formaldehyde was realized via implementing the key enzymes of the ribulose monophosphate (RuMP) pathway from *B. subtilis*: 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase [Bs(Hps) and Bs(Phi)]. Via condensation of formaldehyde and ribulose-5-phosphate (Ru5P) to hexulose-6-phosphate (H6P) and subsequent isomerization to fructose-6-phosphate (F6P), formaldehyde enters the central carbon metabolism. For energy generation, F6P is catabolized via reactions of the glycolysis. Thus it is phosphorylated to fructose-1,6-bisphsophate (FBP) via the phosphofructokinase Pfk and subsequently converted to triosephosphates (TP), which can enter the lower part of glycolysis. For regeneration of the formaldehyde acceptor Ru5P, F6P is either converted via transaldolase and transketolase catalyzed reactions in the non-oxidative part of the pentose phosphate pathway (PPP) or it is isomerized to G6P via the phosphoglucoisomerase Pgi and shuttled into the oxidative part of the PPP. In this part, G6P is converted to 6-phosphoglucolactone (6PGL) by the G6P-dehydrogenase Zwf, which is further converted to 6-phosphogluconate (6PG) by the 6-phosphoglucolactonase Pgl and finally to Ru5P by the 6-phosphogluconate dehydrogenase Gnd. However, recombinant *C. glutamicum* strains were always cultivated on a glucose/methanol mixture and thus, glucose is metabolized as well (**orange**). Glucose uptake and phosphorylation is predominantly performed via the glucose-specific transporter PtsG of the phosphotransferase system yielding glucose-6-phosphate (G6P) and is catabolized via glycolysis and the pentose phosphate pathway.

4.1 Identification and characterization of the endogenous pathway for oxidation of methanol to CO_2

The oxidation of methanol to formaldehyde is the first committed step during methylotrophic growth and the subsequent oxidation of formaldehyde is important to avoid toxic accumulation of this intermediate and to generate additional energy in form of NADH. However, the ability to oxidize C_1 compounds is not exclusively found in methylotrophs, but it is widespread in nature; e.g. it has also been described for the aerobic soil bacterium *P. putida* (Koopman *et al.*, 2009).

4.1.1 Oxidation of methanol by *C. glutamicum* wild type

Although *C. glutamicum* wild type is not able to utilize methanol as sole carbon and energy source, initial growth experiments in medium with glucose and methanol mixtures showed that it is able to oxidize methanol in the stationary phase with a rate of 0.83 ± 0.2 mM/h (2.8 ± 0.5 nmol min^{-1} mg CDW^{-1}). In *C. glutamicum*, methanol oxidation is mainly catalyzed by the NAD^+-dependent alcohol dehydrogenase AdhA, since the methanol consumption rate was reduced by 67% in a strain deficient of *adhA* (0.27 ± 0.05 mM/h). In *C. glutamicum*, four additional genes are

annotated as alcohol dehydrogenases (*adhC*, *adhE*, cg2714, cg0273) (Arndt & Eikmanns, 2007). A contribution of these to the oxidation of methanol could not be shown, since constructed *C. glutamicum* mutants, each carrying a deletion of one of the corresponding genes, showed no reduced methanol oxidation rate in comparison to the wild type. Nevertheless, the effect of the deletion of more than one alcohol dehydrogenase gene in one strain has to be evaluated, since the impact of a single deletion on methanol oxidation might be too small to be detected or since the remaining alcohol dehydrogenases are capable to complement the loss of another one.

The physiological role of AdhA in *C. glutamicum* is the oxidation of ethanol to acetaldehyde during growth on ethanol as carbon source (Arndt & Eikmanns, 2007). Methanol occurs abundantly in nature but it is usually not detectable above 10 μM in the soil (Conrad & Claus, 2005; Kloosterman *et al.*, 2002). Experiments on the response of *C. glutamicum* towards methanol revealed that a concentration of 50 mM decreased the growth rate by about 19% leading to the assumption that a methanol concentration in the range of 10 μM does not affect growth. Thus, it is assumed that the ability to oxidize methanol is not a protection mechanism against toxic concentrations of methanol, but can be mostly attributed to a side activity of the AdhA. The AdhA of *C. glutamicum* strain R, which has 98% sequence identity to AdhA of strain ATCC 13032, was found to have a broad substrate specificity and to be active not only with ethanol, but also with methanol, *n*-propanol, and *n*-butanol. The corresponding specific reaction rates were found to be 7.8, 0.7, 7.7 and 8.9 μmol min^{-1} mg^{-1} (Kotrbova-Kozak *et al.*, 2007). The ability to oxidize methanol via a side activity of a broad-specificity alcohol dehydrogenase was also described for the non-methylotroph *P. putida* (Koopman *et al.*, 2009).

The oxidation of methanol in *C. glutamicum* was shown to be subject to catabolite repression by glucose because oxidation started in the stationary growth phase after glucose has been consumed. With the exception of glutamate and ethanol, the majority of carbon sources are usually co-metabolized with glucose by *C. glutamicum* (Arndt *et al.*, 2008; Krämer *et al.*, 1990; Zahoor *et al.*, 2012). Repression of *adhA* expression by glucose has been described first in studies on ethanol catabolism in *C. glutamicum* (Auchter *et al.*, 2009). The expression of *adhA* is strictly dependent on the activation by the transcriptional regulator RamA, since a *ramA*-deficient strain was no longer able to grow on ethanol as carbon source (Arndt *et al.*, 2008; Auchter *et al.*, 2009; Cramer *et al.*, 2006; Gerstmeir *et al.*, 2004). The deletion mutant *C. glutamicum* Δ*ramA* was

strongly impaired in its ability to oxidize methanol, which is in line with the fact that RamA is essential for transcriptional activation of *adhA*. In addition, DNA-microarray experiments showed that *adhA* expression is up-regulated in the presence of methanol and enzyme assays with cells extracts revealed that the NAD(H)-dependent alcohol dehydrogenase activity of cells cultivated in minimal medium with glucose and methanol was 3-fold higher than in cells solely grown with glucose. Thus, the expression of *adhA* is not only activated in the presence of ethanol, but also in the presence of the C_1 compound methanol.

4.1.2 Oxidation of formaldehyde by *C. glutamicum* wild type

Since formaldehyde is highly cytotoxic and can be found ubiquitously in the environment, the ability for its detoxification is mandatory for most organisms. In *C. glutamicum* it can be generated e.g. via oxidation of methanol via the alcohol dehydrogenase AdhA and/or during growth on vanillate, which is a lignin degradation product (Merkens *et al.*, 2005). In the vanillate catabolism of *C. glutamicum*, the vanillate demethylase/ monooxygenase (encoded by the genes *vanAB*) catalyzes the conversion of vanillate to protocatechuate, in which formaldehyde is released (FIG 8).

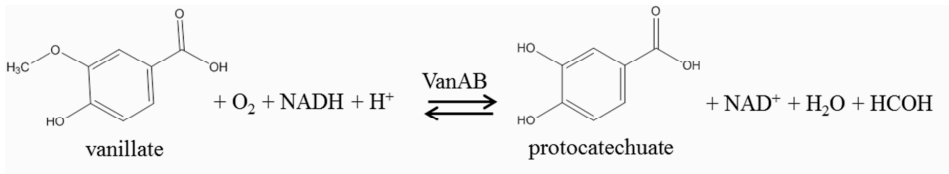

FIG 8 Conversion of vanillate to protocatechuate catalyzed by the vanillate demethylase/ monooxygenase VanAB.

The oxidation of formaldehyde by *C. glutamicum* was shown to be predominantly catalyzed by the enzymes Ald and AdhE, since the wild type showed a formaldehyde oxidation rate of 1.17± 0.2 mM/h, the Δ*ald* mutant a rate of 0.66 ± 0.1 mM/h and the Δ*adhE* mutant a rate of 0.74 ± 0.1 mM/h. The *C. glutamicum* Δ*ald*Δ*adhE* double mutant was almost completely inhibited in its ability to oxidize formaldehyde. Ald is part of ethanol catabolism, where it catalyzes the oxidation of acetaldehyde to acetate (Auchter *et al.*, 2009). Ald of *C. glutamicum* shows 70% sequence identity to the NAD(H)-dependent aldehyde dehydrogenase AldhR from *Rhodococcus erythropolis* UPV-1 (Jaureguibeitia *et al.*, 2007). This enzyme has a broad substrate specificity for aliphatic aldehydes, such as *n*-hexanal, *n*-octanal, and formaldehyde. Hence, it is no surprise

that Ald of *C. glutamicum* also accepts formaldehyde as a substrate. Sequence analyses revealed that AdhE exhibits a high sequence identity of 66% to the MSH-dependent formaldehyde dehydrogenase AdhC of *R. erythropolis* (Eggeling & Sahm, 1985). The mycothiol dependence of AdhE during formaldehyde oxidation in *C. glutamicum* was confirmed by the fact that a mycothiol-defective mutant (Δ*mshC*) showed the same phenotype as a *C. glutamicum* Δ*adhE* mutant and a Δ*ald*Δ*mshC* double mutant behaved similarly to *C. glutamicum* Δ*ald*Δ*adhE*. The findings indicate that *C. glutamicum*, similar to *R. erythropolis* (Yoshida *et al.*, 2011), can oxidize formaldehyde in a MSH-dependent manner via AdhE and in a NAD^+-dependent manner via Ald.

In the case of MSH-dependent oxidation of formaldehyde, the intermediate *S*-formyl-mycothiol is formed (FIG 7A). As the *C. glutamicum* Δ*ald* mutant still accumulates the same amount of formate during growth in methanol containing medium as the wild type, *S*-formyl-mycothiol seems not to be directly oxidized to CO_2 but to be hydrolyzed to formate and mycothiol. The genes cg0388 and cg2193 show sequence similarity to putative S-formyl-MSH hydrolases of *R. erythropolis* and *M. tuberculosis*. However, *C. glutamicum* Δ*ald*Δcg0388 and *C. glutamicum* Δ*ald*Δcg2193 double mutants were unaltered with respect to formate accumulation during growth in methanol containing medium, indicating that the Cg0388 and Cg2193 proteins are not required for hydrolysis of *S*-formyl-mycothiol and that this step might occur spontaneously.

The *C. glutamicum* Δ*ald*Δ*adhE* mutant is still able to oxidize low amounts of formaldehyde and in addition, ^{13}C-methanol labeling experiments revealed that this strain generated only 75% less $^{13}CO_2$/total CO_2 compared to the wild type pointing towards an additional pathway for oxidation of formaldehyde to CO_2 beside Ald and AdhE. In methylotrophs, H_4F and H_4MPT-dependent pathways for formaldehyde oxidation are known (Vorholt, 2002). *C. glutamicum* does not possess the cofactor H_4MPT, but of course H_4F-dependent pathways exist, such as the synthesis of pantothenic acid (Chassagnole *et al.*, 2003). H_4F can spontaneously condense with formaldehyde to form methylene-H_4F. Methylene-H_4F can be oxidized to methenyl-H_4F and subsequently to formyl-H_4F via the bi-functional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase, which is encoded by *folD* (cg0750) in the genome of *C. glutamicum*. The last step of this formaldehyde oxidation pathway

could be catalyzed by the formyl-H_4F deformylase PurU (*purU*, cg0457), which hydrolyzes formyl-H_4F to formate. However, this pathway was not investigated in detail until now.

4.1.3 Oxidation of formate by *C. glutamicum* wild type

Formate is ubiquitously distributed in the environment and plays an important role in microbial metabolism either as substrate for growth or as product of several catabolic reactions (Ferry, 2011; Lin & Iuchi, 1991; Plugge *et al.*, 2011). In *C. glutamicum*, the formate dehydrogenase FdhF and the accessory protein FdhD were identified to confer the capability to oxidize formate stoichiometrically to CO_2 and no other formate-converting enzyme appeared to be present under the conditions tested. Sequence analysis of FdhF revealed conserved residues for binding of a molybdopterin cofactor, which can either bind molybdenum or tungsten to create the biologically active form. In various studies it was demonstrated that the interchange of molybdenum and tungsten led to inactive enzymes (May *et al.*, 1988; McMaster & Enemark, 1998). My results indicate that the FDH from *C. glutamicum* is a molybdenum-dependent enzyme, since the presence of tungstate almost completely inhibited formate consumption by *C. glutamicum*.

The *fdhD* gene of *C. glutamicum* is annotated as an FDH accessory protein. In *E. coli* for example, the FdhD was found to be essential for the activity of three different FDHs (Abaibou *et al.*, 1995; Schlindwein & Mandrand, 1991; Stewart *et al.*, 1991) and it was shown to function as a sulfur transferase between the L-cysteine desulfurase IscS and the molybdenum cofactor present in the active site of the FdhF. This transfer is essential for the sulfur coordination of the molybdenum atom in the molybdenum cofactor (Thome *et al.*, 2012) (FIG 9). FdhD from *E. coli* contains two strictly conserved residues Cys-121 and Cys-124 that are essential for the function of FdhD (Thome *et al.*, 2012). Corresponding cysteine residues are also present within the conserved motif Cys-Gly-Val-Cys-Gly in the FdhD protein from *C. glutamicum* ATCC 13032 (Cys-151, Cys-154) as well as in the proteins of *C. efficiens* YS-314, *M. tuberculosis* H37Rv, *Rhodococcus erythropolis* PR4 and others. It is very likely that FdhD from *C. glutamicum* also acts as a sulfur transferase between a desulfurase and the FdhF to coordinate the molybdenum cofactor and therefore being essential for the formation of an active FDH.

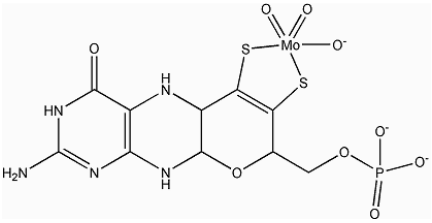

FIG9 Structure of the molybdenum cofactor, which is a complex between molybdopterin and a sulfur-coordinated molybdenum (according to Schwarz *et al.*, (2009)).

Although some characteristics of the FDH from *C. glutamicum* were elucidated, the physiological role of this enzyme is not clear. As carboxylic acids such as formate, acetate and propionate can inhibit bacterial cell growth (Lee *et al.*, 2006; Litsanov *et al.*, 2012; Polen *et al.*, 2003) it was assumed that the ability of *C. glutamicum* to oxidize formate is a protection mechanism against high formate concentrations in the environment. However, growth studies in the presence of formate revealed that a concentration of 10 mM decreased the growth rate only by 5% without any effect on the final optical density. Since formate is mostly found in micromolar concentrations in the soil (Ahumada *et al.*, 2001), it should not affect growth of *C. glutamicum*.

Well-characterized FDHs from various organisms participate in energy generation during aerobic methylotrophic and chemoautotrophic metabolism or are involved in anaerobic fermentative or respiratory processes. As the FDH from *C. glutamicum* is active under aerobic conditions, the involvement in anaerobic processes is quite unlikely. In addition, it could not be shown that the oxidation of formate to CO_2 in *C. glutamicum* is coupled to energy generation, since no *in vitro* activity could be detected in formate dehydrogenase enzyme assays using NAD^+ or $NADP^+$ as cofactors. Thus, seemingly the energy-providing cofactors NADH or NADPH were not generated. However, the involvement of FDH in energy generation cannot be excluded, since electrons could be transferred to other electron acceptors, such as menaquinone or ferredoxins, whose re-oxidation could be coupled with energy conservation. For example, in *C. glutamicum* at least eight different dehydrogenases are known, which transfer the reducing equivalents obtained by oxidation of various substrates, such as succinate or glycerol-3-phosphate, to menaquinone which passes the electrons via the cytochrome bc_1 complex to the

aa$_3$-type cytochrome *c* oxidase or to the cytochrome *bd*-type menaquinol oxidase (Bott & Niebisch, 2003).

For *P. putida* it has been described that FDH, together with its accessory protein, is involved in the formaldehyde detoxification system (Roca *et al.,* 2009). The methylotrophic organism *M. extorquens* possesses four different FDH's, but only FDH4, which has a high sequence identity of 46% to the FdhF of *C. glutamicum*, is essential for oxidation of formate to CO_2 during growth on methanol (Chistoserdova *et al.*, 2007). As shown for FdhF of *C. glutamicum*, this FDH4 is a novel type of FDH with an unknown electron acceptor, which also contains a putative molybdenum-cofactor. The predominant role of the FDH in *C. glutamicum* might be as well the oxidation of intracellular formate, which can be generated e.g. during hydrolysis of 10-formyltetrahydrofolate, in processes involving methanol and/or formaldehyde oxidation or via biosynthesis of riboflavin. Biosynthesis of riboflavin requires a seven-step pathway starting from one molecule GTP and two molecules of ribulose-5-phosphate. This pathway is well studied and an overview is given in Bacher *et al.*, (2000). Formate release occurs during the hydrolytically ring opening of GTP and during rearrangement of ribulose-5-phosphate to 3,4-dihydroxy-2-butanone-4-phosphate, both reactions catalyzed by the bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase, which is encoded by *ribA* in *C. glutamicum* (Takemoto *et al.*, 2014; Volk & Bacher, 1991).

4.2 Engineering of *C. glutamicum* towards methanol utilization

Engineering of *C. glutamicum* towards assimilation of methanol-derived carbon requires a pathway for efficient oxidation of methanol to formaldehyde as well as a pathway for channeling formaldehyde into the central metabolism. Thus, these heterologous pathways were implemented into *C. glutamicum* wild type and *C. glutamicum* Δ*ald*Δ*adhE* followed by characterization of the corresponding recombinant strains regarding their ability to oxidize methanol, to assimilate formaldehyde as well as regarding their growth and biomass formation in the presence of methanol.

4.2.1 Engineering of *C. glutamicum* towards an increased methanol oxidation rate

An initial attempt to improve the methanol oxidation rate of *C. glutamicum* was the overexpression of the endogenous *adhA*, since this alcohol dehydrogenase was shown to be responsible for endogenous oxidation of methanol in *C. glutamicum*. However, the methanol oxidation rate of recombinant strains was not significantly increased. Another approach was the heterologous expression of the gene coding for the NAD^+-dependent Mdh together with the gene coding for the MDH activator protein Act from the methylotroph *B. methanolicus* [Bm(*mdh-act*)]. The NAD^+-dependent Mdh was chosen because PQQ-dependent MDH's are not suitable for an expression in *C. glutamicum* as this organism does not synthesize PQQ or possess any PQQ-dependent enzymes (Shen *et al.*, 2012).

As described for the AdhA of *C. glutamicum* R and *P. putida* (Koopman *et al.*, 2009; Kotrbova-Kozak *et al.*, 2007), the MDHs of *B. methanolicus* show a broad substrate specificity and exhibit higher catalytic activities with ethanol, propanol, butanol, isopropanol and 1,2-propanediol than with methanol. For example, specific activities for the Mdh assayed with these substrates were found to be about 1.35, 1.3, 0.75, 0.3 and 0.1 U/mg, respectively, whereas the activity of Mdh assayed with methanol was about 0.05 U/mg (Krog *et al.*, 2013). These findings indicate that the oxidation of higher alcohols is favored over the oxidation of methanol. It is assumed that the catalytic site of the MDHs of *B. methanolicus* is easily accessible for larger substrates and that the binding of medium-sized primary alcohols as substrates could be more efficient than binding of smaller alcohols such as methanol (Krog *et al.*, 2013). In *B. methanolicus* this problem is circumvented by a high expression of the genes coding for the three different MDH's leading to a high methanol oxidation rate. For the expression of Bm(*mdh-act*) in *C. glutamicum* two different strong promoters were tested: the IPTG-inducible promoter *tac* and the constitutive promoter *tuf*. For unknown reasons, the expression under control of P*tac* led to a negligable *in vitro* activity of Bm(Mdh-Act) in the exponential growth phase and to only a slightly higher one in the stationary phase (0.72 ± 0.2 mU/mg). Nevertheless, the methanol oxidation rate was increased from 0.83 ± 0.1 mM/h to 1.3 ± 0.2 mM/h. In contrast, the expression under control of P*tuf* led to a significantly increased *in vitro* activity of Bm(Mdh-Act) in the exponential growth phase (3.34 ± 1.2 mU/mg) and the methanol oxidation rate of recombinant strains was increased to 1.7 ± 0.3 mM/h over the course of the cultivation. In addition, methanol oxidation was found to set in in

the exponential growth phase. When compared to the average glucose consumption rate during exponential growth of *C. glutamicum* in minimal medium with glucose as carbon source (90 nmol mg^{-1} min^{-1}, (Frunzke *et al.*, 2008), it became obvious that the methanol oxidation rate in the stationary phase of recombinant *C. glutamicum* cells expressing the heterologous methanol dehydrogenase is still quite low (~ 7 nmol mg^{-1} min^{-1}). Same was concluded when comparing the methanol consumption rate of recombinant *C. glutamicum* cells in shake flask experiments (~0.065 g/h) to that of the methylotrophic *B. methanolicus* where under optimized fed-batch conditions the methanol consumption rate is up to 7 g/h (Brautaset *et al.*, 2007). In addition to the optimization of process conditions, methanol oxidation could be improved via engineering of the Mdh towards higher specificity for methanol. A recent publication described that the point mutation S98G in *B. methanolicus* Mdh led to a 3-fold increased V_{max} in comparison to the wild-type enzyme in the absence and the presence of Act (Ochsner *et al.,* 2014). However, the K_m value for methanol (in presence of Act) was significantly increased from 25 ± 9 mM to 847 ± 190 mM and was even higher compared to the wild-type enzyme in absence of Act (349 ± 72 mM). Thus, the catalytic efficiency of MdhS98G was similar to that of the parent enzyme in the absence of Act (Ochsner *et al.*, 2014). To evaluate whether this mutation can be beneficial for our studies, its influence on methanol oxidation has to be evaluated under physiological conditions. In addition, an alcohol dehydrogenase from *Desulfotomaculum kuznetsovii* was identified and found to have a higher activity with methanol than the MDHs from *B. methanolicus* (Ochsner *et al.*, 2014). In the future, this enzyme could be also tested for its use in *C. glutamicum*.

4.2.2 Engineering of *C. glutamicum* wild type towards assimilation of formaldehyde

The *C. glutamicum* wild type was shown to possess an endogenous pathway for the detoxification of naturally occurring cytotoxic formaldehyde to CO_2, but since it is a non-methylotrophic organism and not able to utilize C_1 compounds for biomass generation, a pathway for assimilation of methanol-derived carbon was established. Four different C_1 assimilation pathways in organisms following a methylotrophic lifestyle are known: the Calvin–Benson–Bassham cycle, the serine cycle, the xylulose monophosphate as well as the ribulose monophosphate pathway (Lidstrom, 2006). As the assimilation of C_1 occurs on the level of CO_2 in the Calvin cycle and on the level of CO_2 and methylene-H_4F in the serine cycle, which requires

in both cases the implementation of several enzymes and complex C_1-acceptor regeneration pathways (Smejkalova *et al.*, 2010), we wanted to achieve assimilation on the level of formaldehyde. The assimilation of formaldehyde via the RuMP pathway is more energy-efficient in comparison to the XuMP pathway. Assimilation of 6 mol formaldehyde via the RuMP pathway requires 2 mol ATP and assimilation of 6 mol formaldehyde via the XuMP pathway requires 3 mol ATP. Thus, we established the RuMP pathway via expression of genes encoding for the 3-hexulose-6-phosphate synthase (HPS) and the 6-phospho-3-hexuloisomerase (PHI) in *C. glutamicum*. The RuMP pathway has already been successfully implemented into a few organisms that are used in biotechnological processes. E.g., *hps* and *phi* genes from methylotrophic organisms were successfully transferred to *Burkholderia cepacia* and *Pseudomonas putida* to increase the formaldehyde tolerance and to establish the ability to assimilate formaldehyde, respectively (Koopman *et al.*, 2009; Mitsui *et al.*, 2003; Yurimoto *et al.*, 2009). The expression of *hps* and *phi* genes from the methylotroph *Mycobacterium gastri* resulted in negligible *in vitro* enzyme activity in *C. glutamicum* with 1.40 ± 1.6 mU/mg in the exponential growth phase and 1.02 ± 1.4 mU/mg in the stationary phase. Investigations on the reasons for the low specific activity were not performed. In non-methylotrophic organisms, such as *B. subtilis*, orthologous genes coding for HPS and PHI can be found as well. These enzymes contribute to the detoxification of formaldehyde (Mitsui *et al.*, 2003; Yasueda *et al.*, 1999). Expression of *hps* and *phi* from *B. subtilis* in *C. glutamicum* resulted in active enzymes.

The *C. glutamicum* control strain, harboring the empty vector backbones pVWEx2 and pEKEx2, showed a retarded growth in methanol containing medium and the final backscatter of the culture was reduced by 30% compared to the one reached in medium without methanol. Functional implementation of enzymes for methanol oxidation [Bm(Mdh-Act)] and formaldehyde assimilation [Bs(Hps-Phi)] in the *C. glutamicum* wild type as well as the selection of a suitable promoter for gene expression (P*tuf*), resulted in compensation of this inhibitory effect of methanol. At the end of the exponential growth phase, backscatter measurements of the culture revealed that it grew to a higher density than the control strain in the presence of methanol. This effect might be explained by assimilation of methanol-derived formaldehyde. Indeed, ^{13}C-methanol labeling experiments revealed up to 5% labeling fractions in the m+1 mass isotopomers of various intracellular metabolites in samples taken from the exponential growth

phase. An additional impact might have the generation of NADH due to methanol dehydrogenase catalysed oxidation of methanol in the exponential growth phase and subsequent generation of ATP via oxidative phosphorylation. Thus, less glucose has to be dissimilated for energy generation but can be used for synthesis of biomass precursors. An increased biomass yield on glucose via oxidation of the C_1 compounds formate and formaldehyde was already shown for *Candida utilis* and *Penicillium chrysogenum* (Bruinenberg *et al.*, 1985; Harris *et al.*, 2007) as well as for *P. putida* and a *Saccharomyces cerevisiae* strain expressing a formaldehyde dehydrogenase and a formate dehydrogenase from *Hansenula polymorpha* (Baerends *et al.*, 2008; Koopman *et al.*, 2009).

After exponential growth in glucose/methanol medium, recombinant *C. glutamicum* cells enter a second growth phase, represented by the slightly increasing backscatter of the culture during the "stationary phase" and by an increased final CDW by 5% compared to cultivations without methanol (8.4 ± 0.03 vs. 8.0 ± 0.05 mg/ml). Since the latter effect was not observable during cultivation of a *C. glutamicum* strain, which exclusively expressed the module for methanol oxidation, but not the one for formaldehyde assimilation, the increase in biomass can most probably be attributed to the assimilation of methanol-derived carbon into biomass precursors. Confirmation was obtained by ^{13}C-methanol labeling experiments, since these revealed 3-10% m+1 labeling of selected intracellular metabolites. An inscrutable result represents the high m+1 labeling of the amino acid L-glutamate (labeling fractions of 21% in its m+1 mass isotopomers). In the direct precursor α-ketoglutarate only labeling fractions of 15% in its m+1 mass isotopomers were found, which would point towards a hitherto unknown and quite unlikely reaction to incorporate C_1 compounds into glutamate or to synthesize it via another pathway, not including α-ketoglutarate as precursor. However, labeling fractions of only up to 6% in the m+1 mass isotopomers of L-alanine, L-arginine and L-glutamine, for which L-glutamate serves as precursors, argue against this hypothesis. Thus, with the current knowledge, this result cannot be explained.

Furthermore, the ^{13}C-methanol labeling experiments revealed that the lion's share of methanol is oxidized to CO_2 via the endogenous pathway for formaldehyde dissimilation. Thus, under the conditions tested, the endogenous oxidation of formaldehyde to CO_2 seems to be favored over the assimilation of formaldehyde.

4.2.3 Impact of the absence of the dissimilatory pathway for formaldehyde oxidation on the assimilation of methanol-derived carbon.

Studies on *B. methanolicus* indicated that the fraction of methanol-derived carbon that is dissimilated to CO_2 must be minimized to maximize the biomass yield from methanol (Pluschkell & Flickinger, 2002). In this study, metabolic engineering was used to tackle this problem. The methanol oxidation and formaldehyde assimilation modules were implemented in the *C. glutamicum* Δ*ald*Δ*adhE* mutant, which is devoid of the endogenous pathways for formaldehyde oxidation. As observed for recombinant *C. glutamicum* wild type strains, growth of the recombinant *C. glutamicum* Δ*ald*Δ*adhE* strain in presence of methanol resulted in a 15% higher backscatter at the end of the exponential growth phase compared to the control strain *C. glutamicum* Δ*ald*Δ*adhE* pVWEX2 pEKEx2. As discussed before, this might be explained by the additional NADH generation due to oxidation of methanol. However, in recombinant *C. glutamicum* Δ*ald*Δ*adhE* strains this oxidation has to be followed by channeling the methanol-derived formaldehyde towards the HPS-mediated assimilation, since *C. glutamicum* Δ*ald*Δ*adhE* is missing the "safety valve" for detoxification of formaldehyde via oxidation to CO_2. Without conversion of formaldehyde, toxic amounts of this intermediate would accumulate and inhibit cell growth. In experiments regarding the formaldehyde tolerance of *C. glutamicum* it was shown that the presence of 4 mM formaldehyde led to significant growth retardation. This reasoning is in line with results obtained from ^{13}C-methanol labeling experiments. They revealed that the m+1 labeling of intracellular metabolites in the exponential growth phase was doubled in the recombinant *C. glutamicum* Δ*ald*Δ*adhE* strain in comparison to the *C. glutamicum* wild type strain expressing same genes; in the beginning of the stationary phase it was even three-fold higher. Again, the prominent ^{13}C-labeling of L-glutamate (labeling fractions of 33% in its m+1 mass isotopomers) was striking, but could not be explained.

Although a higher concentration of ^{13}C-labeled intracellular metabolites was observed compared to the recombinant *C. glutamicum* wild type strain expressing the same genes, the elimination of the "safety valve" did not lead to a higher biomass formation in glucose/methanol containing medium. The slower growth, the lower methanol consumption rate (1.25 ± 0.2 mM/h mm/h in comparison to 1.7 ± 0.3 mM/h) and the lower final CDW (6.75 ± 0.01 mg/ml in comparison to 8.37 ± 0.03 mg/ml) indicated towards accumulation of formaldehyde in concentrations that do inhibit

cell growth. This leads to the hypothesis that the synthetic formaldehyde assimilation is not efficient enough to keep the intracellular formaldehyde concentrations below the toxicity threshold.

4.3 Conclusion and Perspective

Implementation of heterologous pathways for methanol oxidation and formaldehyde assimilation into *C. glutamicum* wild type, which harbors an endogenous pathway for dissimilation of formaldehyde, resembles the metabolism from methylotrophic *Bacillus* strains. The detection of ^{13}C-labeled intracellular metabolites during growth in glucose/^{13}C-methanol defined medium (labeling fractions of 3-10% in the m+1 mass isotopomers) as well as the 4% increased CDW in the presence of methanol indicate that methanol-derived carbon was channeled into the central metabolism of *C. glutamicum*. However, an increased methanol oxidation rate is prerequisite for an engineered *C. glutamicum* strain, which is able to grow on methanol as sole carbon and energy source. In contrast to methylotrophic *Bacillus* strains, the lion's share of methanol-derived carbon is currently dissimilated to CO_2. With the aim to minimize this loss of carbon the main pathway for formaldehyde dissimilation in *C. glutamicum* was deleted. This led to an about three-fold increased concentration of ^{13}C-labeled intracellular metabolites, but in comparison to the engineered wild type strain, the final CDW was eventually lower (6.75 ± 0.01 mg/ml vs. 8.37 ± 0.03 mg/ml), probably due to accumulation of formaldehyde in growth inhibiting concentrations. Thus, an important challenge will be the balancing between formaldehyde assimilation and dissimilation, preventing a toxic accumulation of formaldehyde and promoting the assimilation of this C_1 compound. This could be performed via enzyme engineering to improve the affinity of HPS towards formaldehyde or via down-regulation of the endogenous formaldehyde oxidation pathway instead of a simple deletion. A further option to prevent toxic formaldehyde accumulation is an appropriate process control. Since excess methanol promotes the generation of high concentrations of formaldehyde, which is directly detoxified via dissimilation to CO_2, a fed-batch process with continuous feeding of methanol is in interest to develope as it has been realized for *B. methanolicus* MGA3 (Brautaset *et al.*, 2007; Pluschkell & Flickinger, 2002).

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6. Appendix

6.1 Supplementary material "Formate dehydrogenase from *Corynebacterium glutamicum*"

Sequence alignment of known or annotated formate dehydrogenases

```
Corynebacterium glutamicum FdhF 1    ----------MTTPPT--EISNVNP--TANEFDDP--DVGRRITSAAGVP 34
Escherichia coli FDH-H          1    ---------------------------------------------------- 1
Methylobacillus flagellatus KT  88   KVHTQTPKLADIRRGVMELYISDHPLDCLTCSAN-GDCELQDMAGAVGLR 136
Ralstonia eutropha JMP134       88   TVQTESERALRAQRTVLELLQSDMP----ETDYT-RHNELDQWSARLDVG 132
Thermoplasma acidophilum FDH    89   NVVYSEDRVKDLRKEAVQRILANHNLYCTVCDNNNGDCELHNAVLDLKID 138
Methylococcus capsulatus FDH    76   EVRSDSPRARSAQRMVVELLLADQP----ETSRS-RDDELRQWAERLGVS 120
Photobacterium profundum FDH    551  STKERELSFSEVETGFNNDEAMREAARCLECGCQ-ANTDCKLRDYATEYD 599
Methanosarcina barkeri          1    ---------------------------------------------------- 1
Methanobacterium formicium FdhA 1    ---------------------------------------------------- 1
Methanopyrus kandleri           1    ---------------------------------------------------- 1
Mycobacterium bovis FDH-H       1    ----------MYVEAVRWQRSAASR-DVLADYDEQAVTVAPRKREAAGVR 39
Mycobacterium tuberculosis FdhF 1    ----------MYVEAVRWQRSAASR-DVLADYDEQAVTVAPRKREAAGVR 39
Corynebacterium efficiens FDH   1    ----------MTTPP---EISSVNP--LANKFDHP--DVGRRVKSAAGVP 33
Rhodococcus erythropolis        1    ----------MAVPRRSGQYGDMTRQGPSKDIDESDLEVTHPKDYAAGVP 40
Methylobacterium extorquens     1    ----------------------------MDRSQ----GLPKRSSAAGGWG 18
Cupriavidus necator N-1 FdhA    1    ----------------------------MSSPTPEKGHIAPYTHPAAGWG 22

Corynebacterium glutamicum FdhF 35   GVLHALQHAVPNRALLP----LLTMNKPGGIDCPGCAWPEP---------- 71
Escherichia coli FDH-H          1    ---------------------------------------------------- 1
Methylobacillus flagellatus KT  137  EVRYGYDGSNHLHAEKDLSNPYFQFDPSKCIVCSRCVRACEETQG----T 182
Ralstonia eutropha JMP134       133  KPRFA----PRERLAADLSHPAIAVNLDACIQCTRCVRACRDEQVN---- 174
Thermoplasma acidophilum FDH    139  KQKYP---FSRKPYDVDDSNPFYVYDPSQCILCGRCVEACQDVQVN---- 181
Methylococcus capsulatus FDH    121  GSRFP----GREAPPPDRSNPAIAVQLDACIQCTRCVRACRETQVN---- 162
Photobacterium profundum FDH    600  VAETELTNESCQKFHVDDSSEFIVFDANRCISCGQCVEACNEKAVHGTLS 649
Methanosarcina barkeri          1    ---------------------------------------------------- 1
Methanobacterium formicium FdhA 1    ---------------------------------------------------- 1
Methanopyrus kandleri           1    ---------------------------------------------------- 1
Mycobacterium bovis FDH-H       40   AVMVSLQRGMQQMGALRTAAALARLNQRNGFDCPGCAWPE---------- 79
Mycobacterium tuberculosis FdhF 40   AVMVSLQRGMQQMGALRTAAALARLNQRNGFDCPGCAWPE---------- 79
Corynebacterium efficiens FDH   34   GVLHAMEHVVPNRGVLP----LLTMNKPGGFDCPGCAWPEP---------- 70
Rhodococcus erythropolis        41   AVLVSLQRGIEQMGALRTARTLTRLNQRHGFDCPGCAWPE---------- 80
Methylobacterium extorquens     19   ALKSCGKFLLGSRAPISGARALLSANQPDGFDCPGCAWGD---------- 58
Cupriavidus necator N-1 FdhA    23   ALKYVAINLIKEKVAGGKYKMLFKQNQADGFDCPGCAWPD---------- 62

Corynebacterium glutamicum FdhF 71   ---------------------------------------------------- 71
Escherichia coli FDH-H          1    ---------------------------------------------------- 1
Methylobacillus flagellatus KT  183  FALTIQQRGFDSKVAAGNFDN---FLESECVSCGACVTACPTATLMEKTV 229
Ralstonia eutropha JMP134       174  ---DVIGLALRGDAARIVFDMDDPLGASTCVACGECVQACPTGALMPAR- 220
Thermoplasma acidophilum FDH    181  ---ETLHIDWSLERPRVVWDDGSKINESSCVSCGHCVTVCPVNALMEKTM 228
Methylococcus capsulatus FDH    162  ---DVIGYAYRGSHARIVFDQGDPMGLSSCVSCGECVQVCPTGALAPGN- 208
Photobacterium profundum FDH    650  FAKNADGSSASRPECRPGFDKGYSMGDSNCVQCGACVQVCPTGALVDKRD 699
Methanosarcina barkeri          1    ----------------------------------------------------MY 2
Methanobacterium formicium FdhA 1    ---------------------------------------------------- 1
Methanopyrus kandleri           1    ---------------------------------------------------- 1
Mycobacterium bovis FDH-H       79   ---------------------------------------------------- 79
Mycobacterium tuberculosis FdhF 79   ---------------------------------------------------- 79
Corynebacterium efficiens FDH   70   ---------------------------------------------------- 70
Rhodococcus erythropolis        80   ---------------------------------------------------- 80
Methylobacterium extorquens     58   ---------------------------------------------------- 58
Cupriavidus necator N-1 FdhA    62   ---------------------------------------------------- 62
Corynebacterium glutamicum FdhF 71   ----------------------------------------------------ST 73
Escherichia coli FDH-H          1    ----------------------------------------------------MK 2
Methylobacillus flagellatus KT  230  I-------------------------------------EHG--QPEH 237
Ralstonia eutropha JMP134       220  -------------------------------------DAALAVPDQ 229
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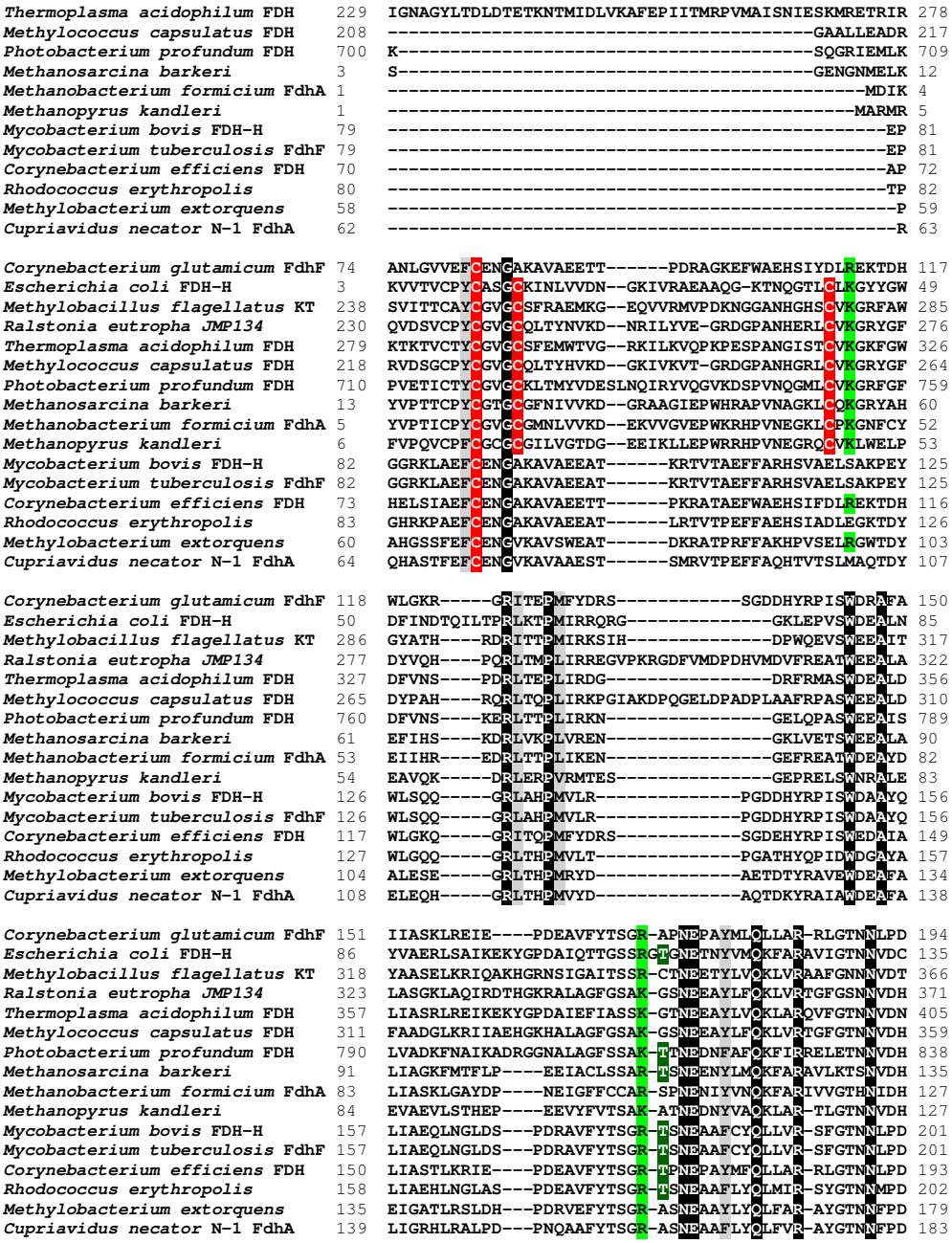
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Photobacterium profundum FDH 700 K----------SQGRIEMLK 709
Methanosarcina barkeri 3 S----------GENGNMELK 12
Methanobacterium formicium FdhA 1 ----------MDIK 4
Methanopyrus kandleri 1 ----------MARMR 5
Mycobacterium bovis FDH-H 79 ----------EP 81
Mycobacterium tuberculosis FdhF 79 ----------EP 81
Corynebacterium efficiens FDH 70 ----------AP 72
Rhodococcus erythropolis 80 ----------TP 82
Methylobacterium extorquens 58 ----------P 59
Cupriavidus necator N-1 FdhA 62 ----------R 63
Corynebacterium glutamicum FdhF 74 ANLGVVEFCENGAKAVAEETT------PDRAGKEFWAEHSIYDLREKTDH 117
Escherichia coli FDH-H 3 KVVTVCPYCASGCKINLVVDN--GKIVRAEAAQG-KTNQGTLCLKGYYGW 49
Methylobacillus flagellatus KT 238 SVITTCAYCGVGCSFRAEMKG--EQVVRMVPDKNGGANHGHSCVKGRFAW 285
Ralstonia eutropha JMP134 230 QVDSVCPYCGVGCQLTYNVKD--NRILYVE-GRDGPANHERLCVKGRYGF 276
Thermoplasma acidophilum FDH 279 KTKTVCTYCGVGCSFEMWTVG--RKILKVQPKPESPANGISTCVKGKFGW 326
Methylococcus capsulatus FDH 218 RVDSGCPYCGVGCQLTYHVKD--GKIVKVT-GRDGPANHGRLCVKGRYGF 264
Photobacterium profundum FDH 710 PVETICTYCGVGCKLTMYVDESLNQIRYVQGVKDSPVNQGMLCVKGRFGF 759
Methanosarcina barkeri 13 YVPTTCPYCGTGCGFNIVVKD--GRAAGIEPWHRAPVNAGKLCQKGRYAH 60
Methanobacterium formicium FdhA 5 YVPTICPYCGVGCGMNLVVKD--EKVVGVEPWKRHPVNEGKLCPKGNFCY 52
Methanopyrus kandleri 6 FVPQVCPFCGCGCGILVGTDG--EEIKLLEPWRRHPVNEGRQCVKLWELP 53
Mycobacterium bovis FDH-H 82 GGRKLAEFCENGAKAVAEEAT------KRTVTAEFFARHSVAELSAKPEY 125
Mycobacterium tuberculosis FdhF 82 GGRKLAEFCENGAKAVAEEAT------KRTVTAEFFARHSVAELSAKPEY 125
Corynebacterium efficiens FDH 73 HELSIAEFCENGAKAVAEETT------PKRATAEFWAEHSIFDLREKTDH 116
Rhodococcus erythropolis 83 GHRKPAEFCENGAKAVAEEAT------LRTVTPEFFAEHSIADLEGKTDY 126
Methylobacterium extorquens 60 AHGSSFEFCENGVKAVSWEAT------DKRATPRFFAKHPVSELRGWTDY 103
Cupriavidus necator N-1 FdhA 64 QHASTFEFCENGVKAVAAEST------SMRVTPEFFAQHTVTSLMAQTDY 107
Corynebacterium glutamicum FdhF 118 WLGKR-----GRITEPMFYDRS----------SGDDHYRPISWDRAFA 150
Escherichia coli FDH-H 50 DFINDTQILTPRLKTPMIRRQRG----------GKLEPVSWDEALN 85
Methylobacillus flagellatus KT 286 GYATH----RDRITTPMIRKSIH----------DPWQEVSWEEAIT 317
Ralstonia eutropha JMP134 277 DYVQH----PQRLTMPLIRREGVPKRGDFVMDPDHVMDVFREATWEEALA 322
Thermoplasma acidophilum FDH 327 DFVNS----PDRLTEPLIRDG----------DRFRMASWDEALD 356
Methylococcus capsulatus FDH 265 DYPAH----RQRLTQPLIRKPGIAKDPQGELDPADPLAAFRPASWEEALD 310
Photobacterium profundum FDH 760 DFVNS----KERLTTPLIRKN----------GELQPASWEEAIS 789
Methanosarcina barkeri 61 EFIHS----KDRLVKPLVREN----------GKLVETSWEEALA 90
Methanobacterium formicium FdhA 53 EIIHR----EDRLTTPLIKEN----------GEFREATWDEAYD 82
Methanopyrus kandleri 54 EAVQK-----DRLERPVRMTES----------GEPRELSWNRALE 83
Mycobacterium bovis FDH-H 126 WLSQQ-----GRLAHPMVLR----------PGDDHYRPISWDAAYQ 156
Mycobacterium tuberculosis FdhF 126 WLSQQ-----GRLAHPMVLR----------PGDDHYRPISWDAAYQ 156
Corynebacterium efficiens FDH 117 WLGKQ-----GRITQPMFYDRS----------SGDEHYRPISWEDAIA 149
Rhodococcus erythropolis 127 WLGQQ-----GRLTHPMVLT----------PGATHYQPIDWDGAYA 157
Methylobacterium extorquens 104 ALESE-----GRLTHPMRYD----------AETDTYRAVEWDEAFA 134
Cupriavidus necator N-1 FdhA 108 ELEQH-----GRLTHPMVYD----------AQTDKYRAIAWDEAFA 138
Corynebacterium glutamicum FdhF 151 IIASKLREIE----PDEAVFYTSGR-APNEPAYMLQLLAR-RLGTNNLPD 194
Escherichia coli FDH-H 86 YVAERLSAIKEKYGPDAIQTTGSSRGTGNETNYVMQKFARAVIGTNNVDC 135
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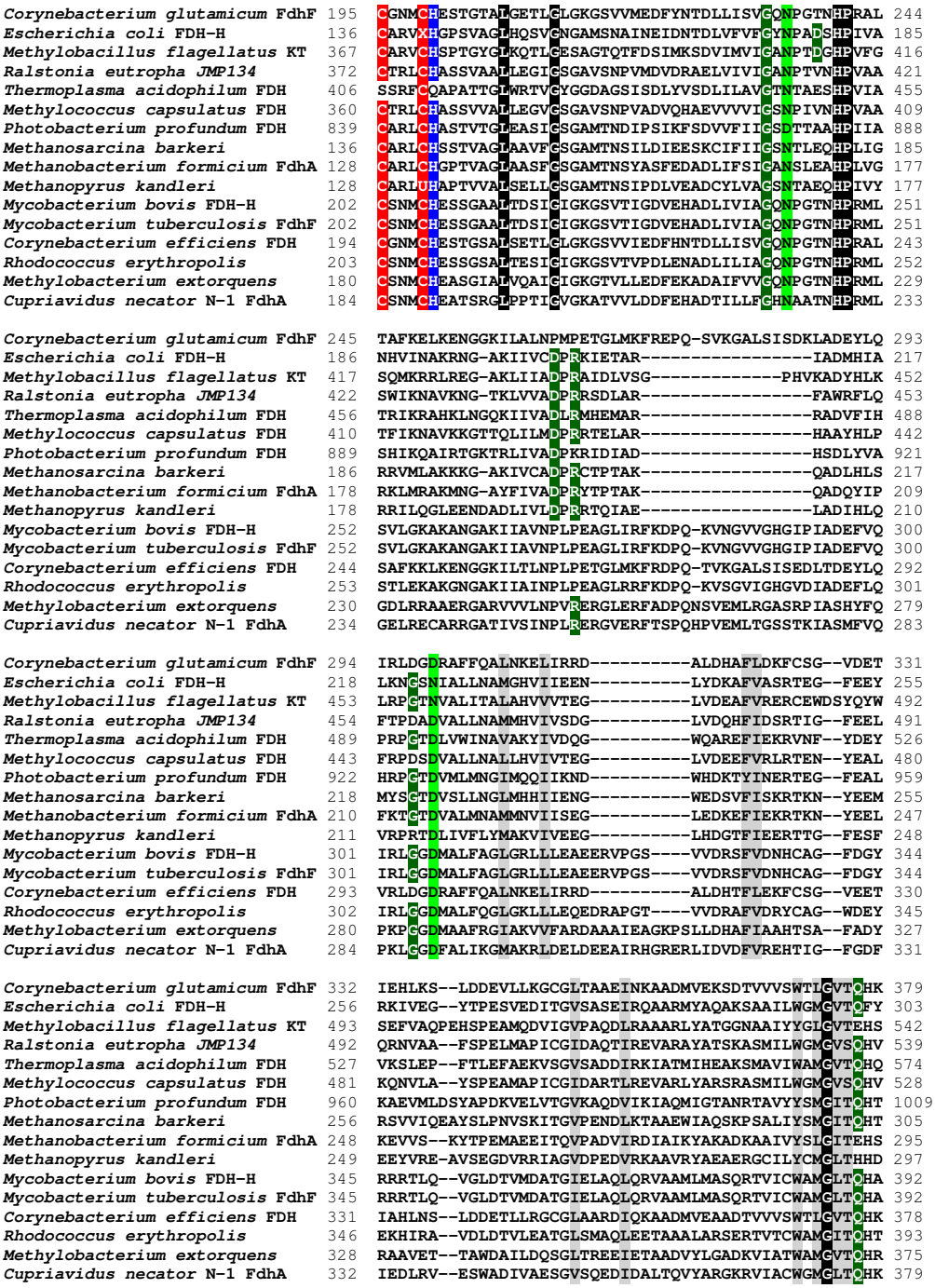
Ralstonia eutropha JMP134 323 LASGKLAQIRDTHGKRALAGFGSAK-GSNEEAYLFQKLVRTGFGSNNVDH 371
Thermoplasma acidophilum FDH 357 LIASRLREIKEKYGPDAIEFIASSK-GTNEEAYLVQKLARQVFGTNNVDN 405
Methylococcus capsulatus FDH 311 FAADGLKRIIAEHGKHALAGFGSAK-GSNEEAYLFQKLVRTGFGTNNVDH 359
Photobacterium profundum FDH 790 LVADKFNAIKADRGGNALAGFSSAK-TTNEDNFAFQKFIRRELETNNVDH 838
Methanosarcina barkeri 91 LIAGKFMTFLP----EEIACLSSAR-TSNEENYLMQKFARAVLKTSNVDH 135
Methanobacterium formicium FdhA 83 LIASKLGAYDP----NEIGFFCCAR-SPNENIYVNQKFARIVVGTHNIDH 127
Methanopyrus kandleri 84 EVAEVLSTHEP----EEVYFVTSAK-ATNEDNYVAQKLAR-TLGTNNVDH 127
Mycobacterium bovis FDH-H 157 LIAEQLNGLDS---PDRAVFYTSGR-TSNEAAFCYQLLVR-SFGTNNLPD 201
Mycobacterium tuberculosis FdhF 157 LIAEQLNGLDS---PDRAVFYTSGR-TSNEAAFCYQLLVR-SFGTNNLPD 201
Corynebacterium efficiens FDH 150 LIASTLKRIE----PDEAVFYTSGR-TPNEPAYMFQLLAR-RLGTNNLPD 193
Rhodococcus erythropolis 158 LIAEHLNGLAS---PDEAVFYTSGR-TSNEAAFLYQLMIR-SYGTNNMPD 202
Methylobacterium extorquens 135 EIGATLRSLDH---PDRVEFYTSGR-ASNEAAYLYQLFAR-AYGTNNFPD 179
Cupriavidus necator N-1 FdhA 139 LIGRHLRALPD---PNQAAFYTSGR-ASNEAAFLYQLFVR-AYGTNNFPD 183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Methylobacillus flagellatus KT 367 CARVCHSPTGYGLKQTLGESAGTQTFDSIMKSDVIMVIGANPTDGHPVFG 416
Ralstonia eutropha JMP134 372 CTRLCHASSVAALLEGIGSGAVSNPVMDVDRAELVIVIGANPTVNHPVAA 421
Thermoplasma acidophilum FDH 406 SSRFCQAPATTGLWRTVGYGGDAGSISDLYVSDLILAVGTNTAESHPVIA 455
Methylococcus capsulatus FDH 360 CTRLCHASSVVALLEGVGSGAVSNPVADVQHAEVVVVIGSNPIVNHPVAA 409
Photobacterium profundum FDH 839 CARLCHASTVTGLEASIGSGAMTNDIPSIKFSDVVFIIGSDTTAAHPIIA 888
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Methanopyrus kandleri 128 CARLUHAPTVVALSELLGSGAMTNSIPDLVEADCYLVAGSNTAEQHPIVY 177
Mycobacterium bovis FDH-H 202 CSNMCHESSGAALTDSIGIGKGSVTIGDVEHADLIVIAGQNPGTNHPRML 251
Mycobacterium tuberculosis FdhF 202 CSNMCHESSGAALTDSIGIGKGSVTIGDVEHADLIVIAGQNPGTNHPRML 251
Corynebacterium efficiens FDH 194 CGNMCHESTGSALSETLGLGKGSVVIEDFHNTDLLISVGQNPGTNHPRAL 243
Rhodococcus erythropolis 203 CSNMCHESSGSALTESIGIGKGSVTVPDLENADLILIAGQNPGTNHPRML 252
Methylobacterium extorquens 180 CSNMCHEASGIALVQAIGIGKGTVLLEDFEKADAIFVVGQNPGTNHPRML 229
Cupriavidus necator N-1 FdhA 184 CSNMCHEATSRGLPPTIGVGKATVVLDDFEHADTILLFGHNAATNHPRML 233
Corynebacterium glutamicum FdhF 245 TAFKELKENGGKILALNPMPETGLMKFREPQ-SVKGALSISDKLADEYLQ 293
Escherichia coli FDH-H 186 NHVINAKRNG-AKIIVCDPRKIETAR----------IADMHIA 217
Methylobacillus flagellatus KT 417 SQMKRRLREG-AKLIIADPRAIDLVSG----------PHVKADYHLK 452
Ralstonia eutropha JMP134 422 SWIKNAVKNG-TKLVVADPRRSDLAR----------FAWRFLQ 453
Thermoplasma acidophilum FDH 456 TRIKRAHKLNGQKIIVADLRMHEMAR----------RADVFIH 488
Methylococcus capsulatus FDH 410 TFIKNAVKKGTTQLILMDPRRTELAR----------HAAYHLP 442
Photobacterium profundum FDH 889 SHIKQAIRTGKTRLIVADPKRIDIAD----------HSDLYVA 921
Methanosarcina barkeri 186 RRVMLAKKKG-AKIVCADPRCTPTAK----------QADLHLS 217
Methanobacterium formicium FdhA 178 RKLMRAKMNG-AYFIVADPRYTPTAK----------QADQYIP 209
Methanopyrus kandleri 178 RRILQGLEENDADLIVLDPRRTQIAE----------LADIHLQ 210
Mycobacterium bovis FDH-H 252 SVLGKAKANGAKIIAVNPLPEAGLIRFKDPQ-KVNGVVGHGIPIADEFVQ 300
Mycobacterium tuberculosis FdhF 252 SVLGKAKANGAKIIAVNPLPEAGLIRFKDPQ-KVNGVVGHGIPIADEFVQ 300
Corynebacterium efficiens FDH 244 SAFKKLKENGGKILTLNPLPETGLMKFRDPQ-TVKGALSISEDLTDEYLQ 292
Rhodococcus erythropolis 253 STLEKAKGNGAKIIAINPLPEAGLRRFKDPQ-KVSGVIGHGVDIADEFLQ 301
Methylobacterium extorquens 230 GDLRRAAERGARVVVLNPVPERGLERFADPQNSVEMLRGASRPIASHYFQ 279
Cupriavidus necator N-1 FdhA 234 GELRECARRGATIVSINPLRERGVERFTSPQHPVEMLTGSSTKIASMFVQ 283
Corynebacterium glutamicum FdhF 294 IRLDGDRAFFQALNKELIRRD----------ALDHAFLDKFCSG--VDET 331
Escherichia coli FDH-H 218 LKNGSNIALLNAMGHVIIEEN----------LYDKAFVASRTEG--FEEY 255
Methylobacillus flagellatus KT 453 LRPGTNVALITALAHVVVTEG----------LVDEAFVRERCEWDSYQYW 492
Ralstonia eutropha JMP134 454 FTPDADVALLNAMMHVIVSDG----------LVDQHFIDSRTIG--FEEL 491
Thermoplasma acidophilum FDH 489 PRPGTDLVWINAVAKYIVDQG----------WQAREFIEKRVNF--YDEY 526
Methylococcus capsulatus FDH 443 FRPDSDVALLNALLHVIVTEG----------LVDEEFVRLRTEN--YEAL 480
Photobacterium profundum FDH 922 HRPGTDVMLMNGIMQQIIKND----------WHDKTYINERTEG--FEAL 959
Methanosarcina barkeri 218 MYSGTDVSLLNGLMHHIIENG----------WEDSVFISKRTKN--YEEM 255
Methanobacterium formicium FdhA 210 FKTGTDVALMNAMMNVIISEG----------LEDKEFIEKRTKN--YEEL 247
Methanopyrus kandleri 211 VRPRTDLIVFLYMAKVIVEEG----------LHDGTFIEERTTG--FESF 248
Mycobacterium bovis FDH-H 301 IRLGGDMALFAGLGRLLLEAEERVPGS----VVDRSFVDNHCAG--FDGY 344
Mycobacterium tuberculosis FdhF 301 IRLGGDMALFAGLGRLLLEAEERVPGS----VVDRSFVDNHCAG--FDGY 344
Corynebacterium efficiens FDH 293 VRLDGDRAFFQALNKELIRRD----------ALDHTFLEKFCSG--VEET 330
Rhodococcus erythropolis 302 IRLGGDMALFQGLGKLLLEQEDRAPGT----VVDRAFVDRYCAG--WDEY 345
Methylobacterium extorquens 280 PKPGGDMAAFRGIAKVVFARDAAAIEAGKPSLLDHAFIAAHTSA--FADY 327
Cupriavidus necator N-1 FdhA 284 PKLGGDFALIKGMAKRLDELDEEAIRHGRERLIDVDFVREHTIG--FGDF 331
Corynebacterium glutamicum FdhF 332 IEHLKS--LDDEVLLKGCGLTAAEINKAADMVEKSDTVVVSWTLGVTQHK 379
Escherichia coli FDH-H 256 RKIVEG--YTPESVEDITGVSASEIRQAARMYAQAKSAAILWGMGVTQFY 303
Methylobacillus flagellatus KT 493 SEFVAQPEHSPEAMQDVIGVPAQDLRAAARLYATGGNAAIYYGLGVTEHS 542
Ralstonia eutropha JMP134 492 QRNVAA--FSPELMAPICGIDAQTIREVARAYATSKASMILWGMGVSQHV 539
Thermoplasma acidophilum FDH 527 VKSLEP--FTLEFAEKVSGVSADDIRKIATMIHEAKSMAVIWAMGVTQHQ 574
Methylococcus capsulatus FDH 481 KQNVLA--YSPEAMAPICGIDARTLREVARLYARSRASMILWGMGVSQHV 528
Photobacterium profundum FDH 960 KAEVMLDSYAPDKVELVTGVKAQDVIKIAQMIGTANRTAVYYSMGITQHT 1009
Methanosarcina barkeri 256 RSVVIQEAYSLPNVSKITGVPENDLKTAAEWIAQSKPSALIYSMGITQHT 305
Methanobacterium formicium FdhA 248 KEVVS--KYTPEMAEEITQVPADVIRDIAIKYAKADKAAIVYSLGITEHS 295
Methanopyrus kandleri 249 EEYVRE-AVSEGDVRRIAGVDPEDVRKAAVRYAEAERGCILYCMGLTHHD 297
Mycobacterium bovis FDH-H 345 RRRTLQ--VGLDTVMDATGIELAQLQRVAAMLMASQRTVICWAMGLTQHA 392
Mycobacterium tuberculosis FdhF 345 RRRTLQ--VGLDTVMDATGIELAQLQRVAAMLMASQRTVICWAMGLTQHA 392
Corynebacterium efficiens FDH 331 IAHLNS--LDDETLLRGCGLAARDIQKAADMVEAADTVVVSWTLGVTQHK 378
Rhodococcus erythropolis 346 EKHIRA--VDLDTVLEATGLSMAQLEETAAALARSERTVTCWAMGITQHT 393
Methylobacterium extorquens 328 RAAVET--TAWDAILDQSGLTREEIETAADVYLGADKVIATWAMGVTQHR 375
Cupriavidus necator N-1 FdhA 332 IEDLRV--ESWADIVAESGVSQEDIDALTQVYARGKRVIACWGMGLTQHK 379

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Corynebacterium glutamicum FdhF  380  NAVYTIREMVNFLLLTGNIGKPGAGTAPLRGHSNVQGDRTMGIW------  423
Escherichia coli FDH-H           304  QGVETVRSLTSLAMLTGNLGKPHAGVNPVRGQNNVQGACDMGALPDTYPG  353
Methylobacillus flagellatus KT   543  QGSTMVMGIANLAMATGNIGREGVGVNPLRGQNNVQGACDMGSFPHEFPG  592
Ralstonia eutropha JMP134        540  HGTDNARCLIALALMTGQLGRPGTGLHPLRGQNNVQGASDAGLIPMMYPD  589
Thermoplasma acidophilum FDH     575  AGSDTSTALSNLLLLTGNYGRPGTGGYPLRGHNNVQGASDFGAMSAYLPG  624
Methylococcus capsulatus FDH     529  HGTDNVRCLIALAMVTGQLGRPGTGLHPLRGQNNVQGASDVGLIPMCFPD  578
Photobacterium profundum FDH     1010 TGHDNVRSVANLQMLCGNIGIEGGGINPLRGQSNVQGACDMGALPNNFPG 1059
Methanosarcina barkeri           306  VGVDNVRSTANLMLLTGNLGVAGGGVNPLRGQNNVQGACDMGCLPDVYPG  355
Methanobacterium formicium FdhA  296  HGVDNVMQTANLAMLTGNIGRLGTGVNPLRGQNNVQGACDMGALPTDYPG  345
Methanopyrus kandleri            298  IATRTVRALCALALLTGNVGRPGTGVNPLRGQNNVQGACDVGALATHFPG  347
Mycobacterium bovis FDH-H        393  HAVATIGEVTNVLLLRGMIGKPGAGVCPVRGHSNVQGDRTMGIW------  436
Mycobacterium tuberculosis FdhF  393  HAVATIGEVTNVLLLRGMIGKPGAGVCPVRGHSNVQGDRTMGIW------  436
Corynebacterium efficiens FDH    379  NAVYTIREMVNFLLLTGNIGKPGAGTAPLRGHSNVQGDRTMGIW------  422
Rhodococcus erythropolis         394  HGVATIEEAVNLLLMRGMMGKPGAGVCPVRGHSNVQGDRTMGIW------  437
Methylobacterium extorquens      376  HSVATIREIANLLFLRGHIGRPGAGLCPVRGHSNVQGDRTVGIN------  419
Cupriavidus necator N-1 FdhA     380  HSVPTVQILSNLMMMRGNIGRPGAGLLPVRGHSNVQGDRTVGIE------  423

Corynebacterium glutamicum FdhF  423  --EKMPEAFLAALENEFGFD-VPRKHGFDTVNSLRAMREG--KTKFFLSL  468
Escherichia coli FDH-H           354  YQYVKDPANREKFAKAWGVESLPAHTGYRISELPHRAAHG--EVRAAYIM  401
Methylobacillus flagellatus KT   593  YRHVSDDTTRALFEAAWGRP-LDKEPGLRIPNMLDFAIHG--SFKALYCE  639
Ralstonia eutropha JMP134        590  YRRVDDPDAIASFEALWGMP-LDRQPGLTVVEIMDAIGRG--EVRGMYIM  636
Thermoplasma acidophilum FDH     625  YQSVSDEKARKKIEEYWKCQ-IPDKPGYDNNTCLEAINSD--RIRAMYVV  671
Methylococcus capsulatus FDH     579  YLRVDDEAARAKFERLWGVP-LDGQPGLTVVEIIDAACAG--RIKGMYIE  625
Photobacterium profundum FDH     1060 YQKVQVPEIHAKFAKAWNKPNLPKEDGLTLTEIIDAACHD--QVKGLYVM 1107
Methanosarcina barkeri           356  YQKVADPENHRKMESIWGVSGLPKAPGLTVTELMEQLAEGTSTVKCMYVM  405
Methanobacterium formicium FdhA  346  YRKVADQEVMEDVTCTWGCSDLGCEPGLKIPEMIDAAAKG--DLKVLYIT  393
Methanopyrus kandleri            348  YRPINTETAN-EMSKIWSFE-VPDEPGLKLTEAFDADEIT-----VMYVV  390
Mycobacterium bovis FDH-H        436  --EKMPEQFLAALDREFGIT-SPRAHGFDTVAAIRAMRDG--RVSVFMGM  481
Mycobacterium tuberculosis FdhF  436  --EKMPEQFLAALDREFGIT-SPRAHGFDTVAAIRAMRDG--RVSVFMGM  481
Corynebacterium efficiens FDH    422  --EKMPESFLQAIEDEFGFD-VPREDGWDTVDSLRAMRDG--KTKFFMSL  467
Rhodococcus erythropolis         437  --EKMPEEFLAALDTEFSIS-SPRKHGWDTVDAIRAMNAG--KASFFMAM  482
Methylobacterium extorquens      419  --EKPPLALLEALDREFGLN-IPRKHGHNVLGAIGAMLDG--SAKAFIGL  464
Cupriavidus necator N-1 FdhA     423  --EKPEQEFLDRLQAAFGFE-PPRKHGYDVVHTISAMLEG--KVKVFVGL  468

Corynebacterium glutamicum FdhF  469  GGNLVRVSSDTSVVEKGMESNELTVHLSTKPNGSQAWPGEQSLILPVIAR  518
Escherichia coli FDH-H           402  GEDPLQTDAELSAVRKAFEDLELVIVQDIFMTKTASAAD---VILPSTSW  448
Methylobacillus flagellatus KT   640  GEDIAQSDPNTQHVTQALSSMECVIVQDLFLNETAMYAH---VFLPGCSF  686
Ralstonia eutropha JMP134        637  GENPAMSDPDAEHAREALAALDHLVVQDIFLTETAYLAD---VVLPASAF  683
Thermoplasma acidophilum FDH     672  GEELVETGSDSEYIRKQLEKLDFLVVEDMFLSETAKYAD---VVLPAAAS  718
Methylococcus capsulatus FDH     626  GENPAMSDPNSNHAREGLASLEHLIVQDLFMTETAYFAD---VILPASAY  672
Photobacterium profundum FDH     1108 GENPVLSDPNQAHVIEGLEKLDFLVVQDIFLTETAQYAD---VVLPSCSF 1154
Methanosarcina barkeri           406  GENFMLSDPDLNKVRKAMKQLDFLVVQDIFLSETANLAD---VVLPAACY  452
Methanobacterium formicium FdhA  394  GEDPVISDPDTHHVEEALNNLDFFVVQDIFMTDTAEFAD---VVLPAACW  440
Methanopyrus kandleri            391  GENPAVSEPNTRHAVEKLESLEFLVVQDLYLTETGELAD---LVLPAAGW  437
Mycobacterium bovis FDH-H        482  GGNFASATPDTAVTEAALRRCALTVQVSTKLNRSHLVHGATALILPTLGR  531
Mycobacterium tuberculosis FdhF  482  GGNFASATPDTAVTEAALRRCALTVQVSTKLNRSHLVHGATALILPTLGR  531
Corynebacterium efficiens FDH    468  GGNLVRVASDTSVLEKGMQSNELTVHVSTKPNGSHAWPGEKSLILPVRAR  517
Rhodococcus erythropolis         483  GGNFIQASPDTAATETALRQCELTVQVSTKLNRSHVVHGQTAIILPTLGR  532
Methylobacterium extorquens      465  GGNFVRATPDTRLVEKALAGCELTVHIATKLNHSHLVPGRVSYLLPCLGR  514
Cupriavidus necator N-1 FdhA     469  GGNFSTATPDTPRTFEALRQCDLTVNIATKLNRSHLVHGKESLILPTLGR  518

Corynebacterium glutamicum FdhF  519  TDKDVQK-SGVQRVTVEDSAGAVHASTGKRTANKDLNLKSECDIIGTIGK  567
Escherichia coli FDH-H           449  GEH------EGVFTAADRGFQRFF-----KAVEPKWDLKTDWQIISEIAT  487
Methylobacillus flagellatus KT   687  LEK------NGTFTNAERRISPVR-----RVMTPK-NGYEDWQITAMLSE  724
Ralstonia eutropha JMP134        684  PEK------TGTFTNTDRTVQLGR-----QALLPPGQARQDLWIIQQMAQ  722
Thermoplasma acidophilum FDH     719  VEK------EGTFVNTERRIQRIY-----RVMEPLGNSRPDWQIIQDVAN  757
Methylococcus capsulatus FDH     673  AEK------TGTFTNTDRIVQIGR-----QAVTPPGEARQDLWIIQEIAR  711
Photobacterium profundum FDH     1155 AEK------SGHFTNTERRVQRIS-----PAVNPPGEAKEDWWIIQSIAN 1193
Methanosarcina barkeri           453  AEK------NGTQTNTERRVQRIR-----KAVDPPGDAKADWRIICELAG  491
Methanobacterium formicium FdhA  441  AEQ------EGTFTNGERRVQLIR-----KAVDAPGESKYDWEIFCDLAK  479
Methanopyrus kandleri            438  AER------TGTFTATDRRVQLAE-----KAVEPPGEARPDWWILEAVAR  476
Mycobacterium bovis FDH-H        532  TDRDTRN-GRKQLVSVEDSMSMVHLSRGSLHPPSDQ-VRSEVQIICQLAR  579
Mycobacterium tuberculosis FdhF  532  TDRDTRN-GRKQLVSVEDSMSMVHLSRGSLHPPSDQ-VRSEVQIICQLAR  579
Corynebacterium efficiens FDH    518  TDRDVQK-TGLQTVTVEDSAGAIHGSTGKRFANRDLDLKSECDVIGSIGR  566
Rhodococcus erythropolis         533  TDLDVQA-GGKQLVSVEDSMSMVHLSRGRLTPVSPY-LRSEVAIICQLAR  580
Methylobacterium extorquens      515  TEIDRNSRAKVQIVTVEDSMSMVHGSGGINKPASPH-LRSEIGIIAGMAA  563
Cupriavidus necator N-1 FdhA     519  TEIDQQD-GVAQGVTVEDSVCMVHISFGMNAPASPH-LLSEIAIVAHMAA  566
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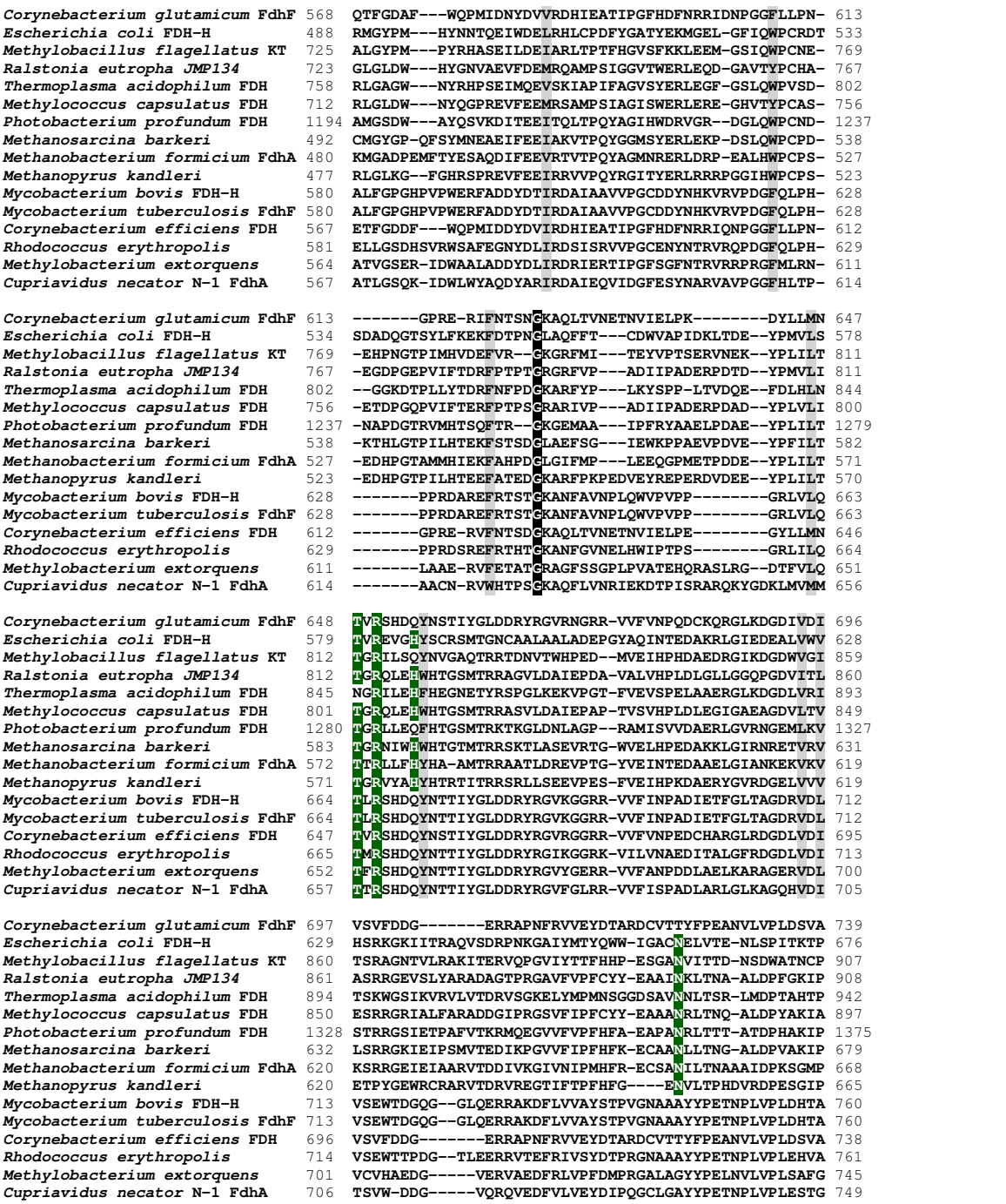
Corynebacterium glutamicum FdhF 568 QTFGDAF---WQPMIDNYDVVRDHIEATIPGFHDFNRRIDNPGGFLLPN- 613
Escherichia coli FDH-H 488 RMGYPM---HYNNTQEIWDELRHLCPDFYGATYEKMGEL-GFIQWPCRDT 533
Methylobacillus flagellatus KT 725 ALGYPM---PYRHASEILDEIARLTPTFHGVSFKKLEEM-GSIQWPCNE- 769
Ralstonia eutropha JMP134 723 GLGLDW---HYGNVAEVFDEMRQAMPSIGGVTWERLEQD-GAVTYPCHA- 767
Thermoplasma acidophilum FDH 758 RLGAGW---NYRHPSEIMQEVSKIAPIFAGVSYERLEGF-GSLQWPVSD- 802
Methylococcus capsulatus FDH 712 RLGLDW---NYQGPREVFEEMRSAMPSIAGISWERLERE-GHVTYPCAS- 756
Photobacterium profundum FDH 1194 AMGSDW---AYQSVKDITEEITQLTPQYAGIHWDRVGR--DGLQWPCND- 1237
Methanosarcina barkeri 492 CMGYGP-QFSYMNEAEIFEEIAKVTPQYGGMSYERLEKP-DSLQWPCPD- 538
Methanobacterium formicium FdhA 480 KMGADPEMFTYESAQDIFEEVRTVTPQYAGMNRERLDRP-EALHWPCPS- 527
Methanopyrus kandleri 477 RLGLKG--FGHRSPREVFEEIRRVVPQYRGITYERLRRRPGGIHWPCPS- 523
Mycobacterium bovis FDH-H 580 ALFGPGHPVPWERFADDYDTIRDAIAAVVPGCDDYNHKVRVPDGFQLPH- 628
Mycobacterium tuberculosis FdhF 580 ALFGPGHPVPWERFADDYDTIRDAIAAVVPGCDDYNHKVRVPDGFQLPH- 628
Corynebacterium efficiens FDH 567 ETFGDDF---WQPMIDDYDVIRDHIEATIPGFHDFNRRIQNPGGFLLPN- 612
Rhodococcus erythropolis 581 ELLGSDHSVRWSAFEGNYDLIRDSISRVVPGCENYNTRVRQPDGFQLPH- 629
Methylobacterium extorquens 564 ATVGSER-IDWAALADDYDLIRDRIERTIPGFSGFNTRVRRPRGFMLRN- 611
Cupriavidus necator N-1 FdhA 567 ATLGSQK-IDWLWYAQDYARIRDAIEQVIDGFESYNARVAVPGGFHLTP- 614
Corynebacterium glutamicum FdhF 613 -------GPRE-RIFNTSNCKAQLTVNETNVIELPK--------DYLLMN 647
Escherichia coli FDH-H 534 SDADQGTSYLFKEKFDTPNCLAQFFT---CDWVAPIDKLTDE--YPMVLS 578
Methylobacillus flagellatus KT 769 -EHPNGTPIMHVDEFVR--CKGRFMI---TEYVPTSERVNEK--YPLILT 811
Ralstonia eutropha JMP134 767 -EGDPGEPVIFTDRFPTPTCRGRFVP---ADIIPADERPDTD--YPMVLI 811
Thermoplasma acidophilum FDH 802 --GGKDTPLLYTDRFNFPDCKARFYP---LKYSPP-LTVDQE--FDLHLN 844
Methylococcus capsulatus FDH 756 -ETDPGQPVIFTERFPTPSCRARIVP---ADIIPADERPDAD--YPLVLI 800
Photobacterium profundum FDH 1237 -NAPDGTRVMHTSQFTR--CKGEMAA---IPFRYAAELPDAE--YPLILT 1279
Methanosarcina barkeri 538 -KTHLGTPILHTEKFSTSDCLAEFSG---IEWKPPAEVPDVE--YPFILT 582
Methanobacterium formicium FdhA 527 -EDHPGTAMMHIEKFAHPDCLGIFMP---LEEQGPMETPDDE--YPLILT 571
Methanopyrus kandleri 523 -EDHPGTPILHTEEFATEDCKARFPKPEDVEYREPERDVDEE--YPLILT 570
Mycobacterium bovis FDH-H 628 -------PPRDAREFRTSTCKANFAVNPLQWVPVPP--------GRLVLQ 663
Mycobacterium tuberculosis FdhF 628 -------PPRDAREFRTSTCKANFAVNPLQWVPVPP--------GRLVLQ 663
Corynebacterium efficiens FDH 612 -------GPRE-RVFNTSDCKAQLTVNETNVIELPE--------GYLLMN 646
Rhodococcus erythropolis 629 -------PPRDSREFRTHTCKANFGVNELHWIPTPS--------GRLILQ 664
Methylobacterium extorquens 611 -------LAAE-RVFETATCRAGFSSGPLPVATEHQRASLRG--DTFVLQ 651
Cupriavidus necator N-1 FdhA 614 -------AACN-RVWHTPSCKAQFLVNRIEKDTPISRARQKYGDKLMVMM 656
Corynebacterium glutamicum FdhF 648 TVRSHDQYNSTIYGLDDRYRGVRNGRR-VVFVNPQDCKQRGLKDGDIVDI 696
Escherichia coli FDH-H 579 TVREVGHYSCRSMTGNCAALAALADEPGYAQINTEDAKRLGIEDEALVWV 628
Methylobacillus flagellatus KT 812 TGRILSQYNVGAQTRRTDNVTWHPED--MVEIHPHDAEDRGIKDGDWVGI 859
Ralstonia eutropha JMP134 812 TGRQLEHWHTGSMTRRAGVLDAIEPDA-VALVHPLDLGLLGGQPGDVITL 860
Thermoplasma acidophilum FDH 845 NGRILEHFHEGNETYRSPGLKEKVPGT-FVEVSPELAAERGLKDGDLVRI 893
Methylococcus capsulatus FDH 801 TGRQLEHWHTGSMTRRASVLDAIEPAP-TVSVHPLDLEGIGAEAGDVLTV 849
Photobacterium profundum FDH 1280 TGRLLEQFHTGSMTRKTKGLDNLAGP--RAMISVVDAERLGVRNGEMLKV 1327
Methanosarcina barkeri 583 TGRNIWHWHTGTMTRRSKTLASEVRTG-WVELHPEDAKKLGIRNRETVRV 631
Methanobacterium formicium FdhA 572 TTRLLFHYHA-AMTRRAATLDREVPTG-YVEINTEDAAELGIANKEKVKV 619
Methanopyrus kandleri 571 TGRVYAHYHTRTITRRSRLLSEEVPES-FVEIHPKDAERYGVRDGELVVV 619
Mycobacterium bovis FDH-H 664 TLRSHDQYNTTIYGLDDRYRGVKGGRR-VVFINPADIETFGLTAGDRVDL 712
Mycobacterium tuberculosis FdhF 664 TLRSHDQYNTTIYGLDDRYRGVKGGRR-VVFINPADIETFGLTAGDRVDL 712
Corynebacterium efficiens FDH 647 TVRSHDQYNSTIYGLDDRYRGVRGGRR-VVFVNPEDCHARGLRDGDLVDI 695
Rhodococcus erythropolis 665 TMRSHDQYNTTIYGLDDRYRGIKGGRK-VILVNAEDITALGFRDGDLVDI 713
Methylobacterium extorquens 652 TFRSHDQYNTTIYGLDDRYRGVYGERR-VVFANPDDLAELKARAGERVDL 700
Cupriavidus necator N-1 FdhA 657 TTRSHDQYNTTIYGLDDRYRGVFGLRR-VVFISPADLARLGLKAGQHVDI 705
Corynebacterium glutamicum FdhF 697 VSVFDDG-------ERRAPNFRVVEYDTARDCVTTYFPEANVLVPLDSVA 739
Escherichia coli FDH-H 629 HSRKGKIITRAQVSDRPNKGAIYMTYQWW-IGACNELVTE-NLSPITKTP 676
Methylobacillus flagellatus KT 860 TSRAGNTVLRAKITERVQPGVIYTTFHHP-ESGANVITTD-NSDWATNCP 907
Ralstonia eutropha JMP134 861 ASRRGEVSLYARADAGTPRGAVFVPFCYY-EAAINKLTNA-ALDPFGKIP 908
Thermoplasma acidophilum FDH 894 TSKWGSIKVRVLVTDRVSGKELYMPMNSGGDSAVNNLTSR-LMDPTAHTP 942
Methylococcus capsulatus FDH 850 ESRRGRIALFARADDGIPRGSVFIPFCYY-EAAANRLTNQ-ALDPYAKIA 897
Photobacterium profundum FDH 1328 STRRGSIETPAFVTKRMQEGVVFVPFHFA-EAPANRLTTT-ATDPHAKIP 1375
Methanosarcina barkeri 632 LSRRGKIEIPSMVTEDIKPGVVFIPFHFK-ECAANLLTNG-ALDPVAKIP 679
Methanobacterium formicium FdhA 620 KSRRGEIEIAARVTDDIVKGIVNIPMHFR-ECSANILTNAAAIDPKSGMP 668
Methanopyrus kandleri 620 ETPYGEWRCRARVTDRVREGTIFTPFHFG----ENVLTPHDVRDPESGIP 665
Mycobacterium bovis FDH-H 713 VSEWTDGQG--GLQERRAKDFLVVAYSTPVGNAAAYYPETNPLVPLDHTA 760
Mycobacterium tuberculosis FdhF 713 VSEWTDGQG--GLQERRAKDFLVVAYSTPVGNAAAYYPETNPLVPLDHTA 760
Corynebacterium efficiens FDH 696 VSVFDDG-------ERRAPNFRVVEYDTARDCVTTYFPEANVLVPLDSVA 738
Rhodococcus erythropolis 714 VSEWTTPDG--TLEERRVTEFRIVSYDTPRGNAAAYYPETNPLVPLEHVA 761
Methylobacterium extorquens 701 VCVHAEDG-----VERVAEDFRLVPFDMPRGALAGYYPELNVLVPLSAFG 745
Cupriavidus necator N-1 FdhA 706 TSVW-DDG-----VQRQVEDFVLVEYDIPQGCLGAYYPETNPLVPLESTG 749

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Corynebacterium glutamicum FdhF  740  EKSNTPVSKSVVVRLEATGRTAS---------------------------- 762
Escherichia coli FDH-H           677  EYKYCAVRVEPIADQRAAEQYVIDEYNKLKTRLREAALA------------ 715
Methylobacillus flagellatus KT   908  EFKVTAVQVTRVSQLSDWQRRYQEFSNSQIALVKQRDMARMG--------- 949
Ralstonia eutropha JMP134        909  EFKYCAIRMTLGGVAPAQSSYGG-GKVLGA--------------------- 937
Thermoplasma acidophilum FDH     943  AYKELPVKMEKIEGGHGESPMPRTNPRYGKPHPQRGVMVEEKWKRSDYIK 992
Methylococcus capsulatus FDH     898  ELKYCAVRVRKGGTVGREFGYQLRGRVA------------------------ 925
Photobacterium profundum FDH     1376 EFKVAAVKIEKVRVLVETC------------------------------- 1394
Methanosarcina barkeri           680  EYKACAVKIEKIEPQEGKLLEEKL--------------------------- 703
Methanobacterium formicium FdhA  669  EYKACAVAISKMEGSK------------------------------------ 684
Methanopyrus kandleri            666  EYKYVPARVRPDSRGSASRG------------------------------- 685
Mycobacterium bovis FDH-H        761  AQSNTPVSKAIIVRLEPTA-------------------------------- 779
Mycobacterium tuberculosis FdhF  761  AQSNTPVSKAIIVRLEPTA-------------------------------- 779
Corynebacterium efficiens FDH    739  EKSNTPASKSVLVRLEPLGVHADDLDK------------------------ 765
Rhodococcus erythropolis         762  AKSNTPVSKAVTVRLEASGA------------------------------- 781
Methylobacterium extorquens      746  EFSDTPTSKSVLVQVRARAANDLGKAA------------------------ 772
Cupriavidus necator N-1 FdhA     750  DGCGTPTSKSVPVLLTPSRRQPAAAA------------------------- 775
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Figure S1 Sequence alignment of known or annotated formate dehydrogenases from *C. glutamicum* (YP_224823.1, cg0618), *E. coli* (NP_418503.1, b4079), *M. flagellatus* KT (YP_544829.1, Mfla_0720), *R. eutropha* JMP 134 (YP_298844.1, Reut_B4651), *T. acidophilum* DSM 1728 (NP_393903.1, Ta0425), *M. capsulatus* (YP_114983.1, MCA2576), *P. profundum* SS9 (YP_132435.1, PBPRB0763), *M. barkeri* (YP_305088.1, Mbar_A1561), *M. formicium* (P06131.1, FDHA_METFO), *M. kandleri* AV19 (NP_613606.1, MK0321), *M. bovis* AF2122/97 (NP_856569.1, Mb2924c), *M. tuberculosis* H37Rv (NP_217416.1, Rv2900c), *C. efficiens* YS-314 (NP_737150.1, CE0540), *R. erythropolis* PR4 (YP_002767252.1, RER_38050), *M. extorquens* AM1 (YP_002963168.1, MexAM1_META1p2094), *C. necator* N-1(YP_004681660.1, CNE_2c14650). The alignment was performed using ClustalW (BioEdit, version 7.0.9.0). Highlighted amino acids: identical (■), similar chemical properties (■), active center (■), conserved cysteine/selenocysteine (■), molybdopterin-binding site and similiar chemical structure (■), molybdopterin-binding site and conserved residue (■).

Amino acid sequence of Cg0618 from *C. glutamicum*

MTTPPTEISNVNPTANEFDDPDVGRRITSAAGVPGVLHALQHAVPNRALLPL[M]LTMNKPGGIDCPGCAWPEP
STANLGVVEFCENGAKAVAEETTPDRAGKEFWAEHSIYDLREKTDHWLGKRGRITEPMFYDRSSGDDHYRPISW
DRAFAIIASKLREIEPDEAVFYTSGRAPNEPAYMLQLLARRLGTNNLPDCGNMCHESTGTALGETLGLGKGSVV
MEDFYNTDLLISVGQNPGTNHPRALTAFKELKENGGKILALNPMPETGLMKFREPQSVKGALSISDKLADEYLQ
IRLDGDRAFFQALNKELIRRDALDHAFLDKFCSGVDETIEHLKSLDDEVLLKGCGLTAAEINKAADMVEKSDTV
VVSWTLGVTQHKNAVYTIREMVNFLLLTGNIGKPGAGTAPLRGHSNVQGDRTMGIWEKMPEAFLAALENEFGFD
VPRKHGFDTVNSLRAMREGKTKFFLSLGGNLVRVSSDTSVVEKGMESNELTVHLSTKPNGSQAWPGEQSLILPV
IARTDKDVQKSGVQRVTVEDSAGAVHASTGKRTANKDLNLKSECDIIGTIGKQTFGDAFWQPMIDNYDVVRDHI
EATIPGFHDFNRRIDNPGGFLLPNGPRERIFNTSNGKAQLTVNETNVIELPKDYLLMNTVRSHDQYNSTIYGLD
DRYRGVRNGRRVVFVNPQDCKQRGLKDGDIVDIVSVFDDGERRAPNFRVVEYDTARDCVTTYFPEANVLVPLDS
VAEKSNTPVSKSVVVRLEATGRTAS

Figure S2 Deduced amino acid sequence of Cg0618 from *C. glutamicum*. The first methionine (M) represents the translational start given by the annotation by Kalinowski *et al*. (2003) and the second methionine ([M]) represents the translational start given by the annotation by Ikeda and Nakagawa (2003). The tryptic peptides found by MALDI-ToF-MS (Table S1) with purified and digested FdhF protein expressed from a plasmid with the native promotor region of cg0618 cover the sequences highlighted in grey. The covering of the N-terminal sequence deduced from the annotation by Kalinowski et al. (2003) reflects that cg0618 is translated according to Kalinowski *et al*. (2003) and the initial methionine is cleaved off.

Table S1 Masses measured in a MALDI-TOF MS peptide mass fingerprint analysis of a tryptic digest of purified Cg0618 from *C. glutamicum* matching to 31 peptides of Cg0618 covering 43% of the NCBI protein sequence. The peptides are sorted by their position in the protein given by the amino acid residue numbers.

Amino acid position	Observed	M_r (expected)	M_r (calculated)	p.p.m	Miss	Sequence
2-26	2742.3259	2741.3186	2741.2893	11	1	R.ITSAAGVPGVLHALQHAVPNR.A
27-47	2108.2182	2107.2109	2107.1651	22	0	M.TTPPTEISNVNPTANEFDDPDVGRR.I
98-112	1821.9351	1820.9278	1820.8846	24	1	R.AGKEFWAEHSIYDLR.E
101-112	1565.7783	1564.7710	1564.7310	26	0	K.EFWAEHSIYDLR.E
125-133	1187.5702	1186.5629	1186.5329	25	0	R.ITEPMFYDR.S Ox (M)
134-147	1690.7996	1689.7924	1689.7495	25	0	R.SSGDDHYRPISWDR.A
156-171	1881.9772	1880.9700	1880.9268	23	1	K.LREIEPDEAVFYTSGR.A
158-171	1612.7868	1611.7795	1611.7417	23	0	R.EIEPDEAVFYTSGR.A
172-185	1586.8736	1585.8663	1585.8286	24	0	R.APNEPAYMLQLLAR.R
172-185	1602.8687	1601.8615	1601.8235	24	0	R.APNEPAYMLQLLAR.R Ox (M)
216-242	2960.4581	2959.4508	2959.4134	13	0	K.GSVVMEDFYNTDLLISVGQNPGTNHPR.A
216-242	2976.4551	2975.4478	1749.8058	13	0	K.GSVVMEDFYNTDLLISVGQNPGTNHPR.A Ox (M)
279-295	1892.0596	1891.0523	1891.0051	25	1	K.GALSISDKLADEYLQIR.L
287-295	1120.6302	1119.6229	1119.5924	27	0	K.LADEYLQIR.L
387-409	2369.3380	2368.3307	2368.2937	16	0	R.EMVNFLLLTGNIGKPGAGTAPLR.G
387-409	2385.3371	2384.3298	2384.2886	17	0	K.MPEAFLAALENEFGFDVPR.K Ox (M)
426-444	2169.0803	2168.0731	2168.0248	22	0	R.EMVNFLLLTGNIGKPGAGTAPLR.G Ox (M)
445-455	1273.6985	1272.6912	1272.6575	26	1	K.DGDIVDIVSVFDDGER.R
462-474	1451.8759	1450.8686	1450.8296	27	1	K.TKFFLSLGGNLVR.V
464-474	1750.8510	1221.7216	1221.6870	28	0	K.FFLSLGGNLVR.V
568-586	2318.0955	2317.0882	2317.0474	18	0	K.QTFGDAFWQPMIDNYDVVR.D Ox (M)
587-601	1768.8832	1767.8759	1767.8329	24	0	R.DHIEATIPGFHDFNR.R
587-602	1924.9880	1923.9807	1923.9340	24	1	R.DHIEATIPGFHDFNRR.I
602-616	1622.9176	1621.9104	1621.8689	26	1	R.RIDNPGGFLLPNGPR.E
603-616	1466.8132	1749.8437	1465.7678	26	0	R.IDNPGGFLLPNGPR.E
627-650	2790.5121	2789.5048	2789.4633	15	1	R.SHDQYNSTIYGLDDR.Y
651-665	1783.8328	1782.8255	1782.7809	25	0	K.AQLTVNETNVIELPKDYLLMNTVR.S Ox (M)
651-667	2102.9946	2101.9874	2101.9453	20	1	R.SHDQYNSTIYGLDDRYR.G
675-686	1489.7854	1488.7781	1488.7507	18	1	R.VVFVNPQDCKQR.G
687-705	2049.0571	2048.0499	2048.0062	21	1	R.GLKDGDIVDIVSVFDDGER.R
690-705	1222.7289	1749.8437	2975.4084	22	0	R.KHGFDTVNSLR.A

6.2 Supplementary material "Endogenous methanol oxidation in *Corynebacterium glutamicum*"

Table S1 Oligonucleotides used in this study

Oligonucleotide	Sequence (5' → 3') and properties [a]
Construction and verification of the Δ*adhA* mutation	
P_Δ*adhA*_1	CTG AGT CGA CAC GAT CCT TGG AAT GTT GTT G (SalI)
P_Δ*adhA*_2	*CCC ATC CAC TAA ACT TAA ACA* TTC TTG GGG TGC AGC AGT GGT CAT
P_Δ*adhA*_3	*TGT TTA AGT TTA GTG GAT GGG* GGT GTG CTT GAC CGC ATG CGA
P_Δ*adhA*_4	CAG TAA GCT TGA AAG TTG TCA CTG CGC AAC (HindIII)
P_Δ*adhA*_out_fw	AGT GCC TTA AGC ACC GGC AG
P_Δ*adhA*_out_rev	CAG CTG ATA CAG CTC TGA GG
Construction and verification of the Δ*adhC* mutation	
P_Δ*adhC*_1	CTG ACC CGG GAT CCA ACT TTG CCG TGG TAG (XmaI)
P_Δ*adhC*_2	*CCC ATC CAC TAA ACT TAA ACA* TGC TTT TAC TGA GAT ACT CAC CCC
P_Δ*adhC*_3	*TGT TTA AGT TTA GTG GAT GGG* GAC GTT CAG TTC CGC GTT GTC ATT
P_Δ*adhC*_4	CAG TTC TAG ACA AAT ATC GAT TGA GCA CGC (XbaI)
P_Δ*adhC*_out_fw	ATG AGG TCT CCG ATC CTG AG
P_Δ*adhC*_out_rev	TTG GAG GAT TGG TTT CCT TG
Construction and verification of the Δ*ald* mutation	
P_Δ*ald*_1	AG TGG ATC CCC GAA ACC TCA AAG AAT CCC (BamHI)
P_Δ*ald*_2	*CC ATC CAC TAA ACT TAA ACA* TCC TGG ATT TGC GTA GAC AGT CAT
P_Δ*ald*_3	*GT TTA AGT TTA GTG GAT GGG* TAC CAG CAG ACC AAG AAC CTG TTG
P_Δ*ald*_4	AG TAA GCT TGA TCT CCA GAG GTT TCA AGC (HindIII)
P_Δ*ald*_out_fw	AA TCA AGG CGA TCA TCG AG
P_Δ*ald*_out_rev	CG ATT CCT GCT GCG GTA TC
Construction and verification of the Δ*adhE* mutation	
P_Δ*adhE*_1	CTG ACC CGG GCA GAA GCA GAT CTT GCA ATC (XmaI)
P_Δ*adhE*_2	*CCC ATC CAC TAA ACT TAA ACA* AAT TCC AGG CAC TAC AGT GCT CAT
P_Δ*adhE*_3	*TGT TTA AGT TTA GTG GAT GGG* GAA GAG GCT TTC AAC ACC ATG AAG
P_Δ*adhE*_4	CAG TAA GCT TGG AAC AAA GTG TCT CCA GAG (HindIII)
P_Δ*adhE*_out_fw	ACA GCT GGA AGC AGC CAT TC
P_Δ*adhE*_out_rev	GAC TCA ATG ATG GTG TCA AAG G
Construction and verification of the Δcg2714 mutation	
P_Δcg2714_1	CTG ACC CGG GCC TGA CAC TCA GAT CGG AC (SmaI)
P_Δcg2714_2	*CCC ATC CAC TAA ACT TAA ACA* AAC AAT AGC AGC AAG GGT TTG CAT
P_Δcg2714_3	*TGT TTA AGT TTA GTG GAT GGG* GTC AAC ATT GAT AAC GGC TAT G
P_Δcg2714_4	CTG ATC TAG ACG ATT TCT CCA CAA TCA AGG C (XbaI)
P_Δcg2714_out_fw	TGC ATT CAC CGC TTT CGC T
P_Δcg2714_out_rev	AAG GCA CCA CCG AGT AAC

Construction and verification of the Δ*cg0273* mutation

P_Δcg0273_1	AGC TCT AGA TAC TAG GTC GTG TGC TGT GG (XbaI)
P_Δcg0273_2	*CCC ATC CAC TAA ACT TAA ACA* CTG CAT GGC AAT GTA TTT GGG CAT
P_Δcg0273_3	*TGT TTA AGT TTA GTG GAT GGG* GCT ATT GCA CGT ATT TCA GCT GGT
P_Δcg0273_4	AGC AAG CTT CCT CAC TGA GAG AAT GGA CG (HindIII)
P_Δcg0273_out_fw	CCT GCA AGA AGT CCT TCA TG
P_Δcg0273_out_rev	GCT TTG CCC TCA CAA TCT G

Construction and verification of the Δ*cg0388* mutation

P_Δcg0388_1	CTG ATC TAG AGC CAA GAT TGC TGG CGC TTC (XbaI)
P_Δcg0388_2	*CCC ATC CAC TAA ACT TAA ACA* GCG CAA TCC GTC GTG AGC CAT
P_Δcg0388_3	*TGT TTA AGT TTA GTG GAT GGG* GCT GAG GCT CCA CAC TTG GAG
P_Δcg0388_4	CTG AAA GCT TCC GCC AAA TTT TTC ACC ACG AC (HindIII)
P_Δcg0388_out_fw	AGT CCG TAG CAG TCT TCG
P_Δcg0388_out_rev	GCT CAG CTA CCA CGA ATC

Construction and verification of the Δ*cg2193* mutation

P_Δcg2193_1	CTG AGG ATC CGG CTC CAG CTG ACG CAG C (BamHI)
P_Δcg2193_2	*CCC ATC CAC TAA ACT TAA ACA* CCA CTG CTG CAT CTG ATT CAT
P_Δcg2193_3	*TGT TTA AGT TTA GTG GAT GGG* GAA GTA CTC AAC AAC TGG CTG CA
P_Δcg2193_4	CTG ACT GCA GCA CGT CGA TAA GCC TCG CC (PstI)
P_Δcg2193_out_fw	TGT AGT TCA GCT CGC ATA GAG
P_Δcg2193_out_rev	CGA TGC GTG CAT GTC ATA CA

Verification of the Δ*mshC* mutation

P_Δ*mshC*_out_fw	GAT CTA CAA TTG GAC AAG CTG CCT CCG GCC TA [b]
P_Δ*mshC*_out_rev	GAT CTA GGA TCC GCT CGA TTG CTC GTT GGT TA [b]

Plasmid-based expression of *adhA* for complementation studies (pAN6-*adhA*)

P_*adhA*_fw_NdeI	CAG TCA TAT GAC CAC TGC TGC ACC CCA AG
P_*adhA*_rev_NheI	GAC TGG CTA GCT TAG AAA CGA ATC GCC ACA CGA TT

Plasmid-based expression of *ald* for complementation studies (pAN6-*ald*)

P_*ald*_fw_NdeI	CTG ACA TAT GAC TGT CTA CGC AAA TCC AG
P_*ald*_rev_NheI	CTG AGC TAG CTC AGA ACA GTC CGG TTG GGT

Plasmid-based expression of *adhE* for complementation studies (pAN6-*adhE*)

P_*adhE*_fw_NdeI	CTG ACA TAT GAG CAC TGT AGT GCC TGG
P_*adhE*_rev_NheI	CTG AGC TAG CTT AGA TCT CCA CCA CAG AAC G

Plasmid-based expression of *ald* and *adhE* for complementation studies (pAN6-*ald-adhE*)

P_*ald*_fw_NdeI	CTG ACA TAT GAC TGT CTA CGC AAA TCC AG
P_*ald*_rev_NheI	CTG AGC TAG CTC AGA ACA GTC CGG TTG GGT
P_*adhE*_fw_NheI	CTG AGC TAG CGG AGG AGA TAT AGC TAT GAG CAC TGT AGT GCC
P_*adhE*_rev_NheI	CTG AGC TAG CTT AGA TCT CCA CCA CAG AAC G

Sequencing of *adhA*, *ald* and *adhE* in pAN6

P_ pEKEx-for2	CGG CGT TTC ACT TCT GAG TTC GGC
P_ pEKEx-rv2	GAT ATG ACC ATG ATT ACG CCA AGC

[a] Recognition sites for the indicated restriction enzymes are underlined. Nucleotides shown in italic represent the complementary sequences used for crossover-PCR.

[b] Sequences were taken from Feng *et al*. (2006).

Table S2. Genome-wide comparison of mRNA levels in *C. glutamicum* wild type during growth in CGXII minimal medium supplemented with 2% glucose and 100 mM methanol versus growth in CGXII minimal medium supplemented with 2% glucose.[a)]

Locus tag	Gene and/or annotation	mRNA ratio $+CH_3OH/-CH_3OH$	p value
cg2560	*aceA*, isocitrate lyase	113.0	6.58E-03
cg2559	*aceB*, malate synthase	32.3	3.01E-03
cg3047	*ackA*, acetate/propionate kinase	11.5	6.04E-05
cg3048	*pta*, phosphotransacetylase	9.3	5.56E-07
cg3107	*adhA*, Zn-dependent alcohol dehydrogenase	4.4	1.93E-15
cg0949	*gltA*, citrate synthase	4.1	5.34E-08
cg2558	related to aldose 1-epimerase	3.5	2.21E-13
cg0952	putative integral membrane protein	3.4	7.41E-03
cg0791	*pyc*, pyruvate carboxylase	3.2	1.22E-24
cg0953	*mctC*, monocarboxylate transporter	3.0	9.30E-17
cg0762	*prpC2*, 2-methylcitrate synthase	2.5	2.82E-03
cg2561	*thiX*, protein potentially involved into thiamin biosynthesis	2.5	1.11E-04
cg0759	*prpD2*, 2-methylcitrate dehydratase	2.4	3.27E-03
cg0760	*prpB2*, methylisocitrate lyase	2.4	1.60E-04
cg0690	*groES*, chaperonin	2.4	2.02E-14
cg1701	*metH*, homocysteine methyltransferase	2.4	6.01E-07
cg0754	*metA*, homoserine O-acetyltransferase	2.3	1.63E-09
cg1739	glutamine amidotransferase	2.2	1.38E-09
cg3096	*ald*, acetaldehyde dehydrogenase	2.1	3.87E-10
cg1435	*ilvB*, acetolactate synthase, large subunit	0.47	2.15E-15
cg0898	pyridoxine biosynthesis protein	0.45	3.50E-06
cg0899	glutamine AT involved in pyridoxine biosynthesis	0.45	1.05E-13
cg0598	*rplB*, 50S ribosomal protein	0.44	1.92E-04
cg2925	*ptsS*, enzyme II for sucrose uptake	0.44	2.54E-18
cg1879	HIT family hydrolase	0.42	1.47E-03
cg0116	*ureE*, urease accessory protein	0.42	3.10E-06
cg1215	*nadC*, nicotinate-nucleotide pyrophosphorylase	0.41	9.35E-04
cg1216	*nadA*, quinolinate synthetase	0.41	2.27E-02
cg0576	*rpoB*, DNA-directed RNA polymerase beta subunit	0.41	2.92E-21
cg3226	putative L-lactate permease	0.37	9.20E-07
cg2162	thymidylate synthase	0.36	4.28E-02
cg0770	siderophore ABC transporter, permease protein	0.34	2.77E-02
cg0771	secreted siderophore-binding lipoprotein	0.22	1.14E-01
cg0117	*ureF*, urease accessory protein	0.17	1.48E-01
cg3335	*malE*, malic enzyme	0.14	5.31E-07
cg1214	cysteine sulfinate desulfinase/cysteine	0.14	1.46E-01
cg2836/7	*sucD/C*, succinyl-CoA synthetase alpha/beta subunit	0.04	1.58E-01

[a)] Three biological replicates were performed. To evaluate and filter the generated data, the signal-to-noise ratio for the individual data points was set to > 5 and just genes regulated more than two-fold were considered. Listed are all genes which were regulated in at least two of the three performed experiments with their average ratio of medians and p-values. Genes with a p-value < 0.05 are considered as significant.

Table S3 Genome-wide comparison of mRNA levels of resting *C. glutamicum* cells incubated in CGXII minimal medium with 100 mM methanol as sole carbon source versus without any carbon source. [a)]

Locus tag	Gene and/or annotation	mRNA ratio +CH_3OH/-CH_3OH	p value
cg2560	*aceA*, isocitrate lyase	3922.61	1.45E-01
cg1581	*argJ*, bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	1320.08	7.49E-02
cg1585	*argR*, arginine repressor	693.63	2.35E-02
cg0952	putative integral membrane protein	478.23	1.62E-01
cg3107	*adhA*, Zn-dependent alcohol dehydrogenase	456.00	1.05E-01
cg1583	*argD*, acetylornithine aminotransferase	360.59	1.48E-01
cg0230	*gltD*, glutamine 2-oxoglutarate aminotransferase small SU	320.61	1.77E-01
cg3047	*ackA*, acetate/propionate kinase	194.75	1.52E-01
cg1227	*ykoE*, substrate-specific component YkoE of thiamin-regulated ECF transporter for hydroxymethylpyrimidine	157.04	1.59E-01
cg1434	*yggB*, small-conductance mechanosensitive channel	130.42	1.84E-01
cg1108	*porC*, putative secreted protein	129.31	1.44E-01
cg0825	*fabG*, 3-ketoacyl-(acyl-carrier-protein) reductase	117.84	9.87E-02
cg1580	*argC*, N-acetyl-gamma-glutamyl-phosphate reductase	95.05	1.51E-01
cg0472	hypothetical protein cg0472	89.98	1.35E-01
cg1586	*argG*, argininosuccinate synthase	82.04	1.98E-01
cg0173	hypothetical protein cg0173	81.42	2.20E-01
cg1911	putative secreted protein	81.23	1.68E-01
cg0078	hypothetical protein cg0078	81.00	1.60E-01
cg1556	hypothetical protein cg1556	78.01	2.25E-01
cg0994	*rpmE*, 50S ribosomal protein L31	77.57	1.90E-01
cg3432	*rpmH*, 50S ribosomal protein L34	76.57	1.88E-01
cg1333	*argS*, arginyl-tRNA synthetase	74.76	1.61E-01
cg0133	p-aminobenzoyl-glutamate transporter	72.81	2.23E-01
cg1564	*rpmI*, 50S ribosomal protein L35	72.76	2.07E-01
cg1123	*greA*, transcription elongation factor GreA	67.69	1.86E-01
cg0077	hypothetical protein cg0077	65.44	1.53E-01
cg0953	*mctC*, monocarboxylic acid transporter	60.64	7.83E-02
cg1072	*rplY*, 50S ribosomal protein L25	60.16	2.20E-01
cg2167	*rpsO*, 30S ribosomal protein S15	58.70	1.89E-01
cg0948	*serC*, phosphoserine aminotransferase	55.31	2.00E-01
cg0107	secreted protein	54.59	2.27E-01
cg3114	*cysN*, sulfate adenyltransferase subunit 1	51.05	1.43E-01
cg2887	*phoS*, two component sensor kinase	50.65	2.22E-01
cg1662	putative secreted protein	49.10	2.02E-01
cg0989	*rpsN*, 30S ribosomal protein S14	49.03	2.26E-01
cg0188	hypothetical protein cg0188	47.20	2.64E-01
cg2561	*thiX*, protein potentially involved into thiamin biosynthesis	45.82	2.48E-01
cg2052	putative secreted protein	44.84	1.87E-01
cg3116	*cysH*, phosphoadenosine-phosphosulfate reductase	42.97	1.58E-01
cg0493	hypothetical protein predicted by Glimmer	42.96	1.98E-01
cg0291	3,4-dioxygenase beta subunit	41.10	1.92E-01

cg1841	*aspS*, aspartyl-tRNA synthetase	39.96	1.87E-01
cg0811	*dtsR2*, acetyl/propionyl CoA carboxylase, beta subunit	38.96	2.25E-01
cg1433	hypothetical protein cg1433	38.54	2.41E-01
cg1910	putative secreted or membrane protein	38.13	2.75E-01
cg2096	hypothetical protein cg2096	37.57	2.73E-01
cg3386	*tcbF*, maleylacetate reductase	36.69	2.12E-01
cg1584	*argF*, ornithine carbamoyltransferase	35.21	2.51E-01
cg2492	*glmS*, D-fructose-6-phosphate amidotransferase	34.94	2.47E-01
cg0783	hypothetical protein cg0783	34.21	2.08E-01
cg0629	*rplF*, 50S ribosomal protein L6	33.50	2.14E-01
cg2834	*cysE*, serine O-acetyltransferase	33.14	2.40E-01
cg0674	*rpsI*, 30S ribosomal protein S9	32.87	2.40E-01
cg3385	catA3, catechol 1,2-dioxygenase	32.82	1.83E-01
cg2603	*ndk*, nucleoside diphosphate kinase	32.51	2.59E-01
cg3048	*pta*, phosphate acetyltransferase	32.01	2.21E-02
cg1787	*ppc*, phosphoenolpyruvate carboxylase	31.24	2.07E-01
cg2468	branched-chain amino acid ABC-type transport system,	30.92	2.11E-01
cg0535	probable ketoglutarate semialdehyde dehydrogenase	30.29	2.49E-01
cg1762	*sufC*, Fe-S cluster assembly ATPase	29.42	1.79E-01
cg0422	*murA*, UDP-N-acetylglucosamine 1-carboxyvinyltransferase	29.36	2.59E-01
cg0608	*rplN*, 50S ribosomal protein L14	29.25	2.23E-01
cg1055	*menG*, ribonuclease activity regulator protein RraA	28.89	2.73E-01
cg1894	hypothetical protein cg1894	28.85	2.82E-01
cg1226	*pobB*, 4-hydroxybenzoate 3-monooxygenase	28.22	2.45E-01
cg0949	*gltA*, citrate synthase	27.89	3.77E-04
cg0536	putative 5-dehydro-4-deoxyglucarate dehydratase	27.87	2.39E-01
cg1559	Zn-dependent hydrolase	27.73	2.46E-01
cg3190	membrane-associated phospholipid phosphatase	27.58	2.29E-01
cg0203	*iolE*, 2-Keto-myo-inositol dehydratase	26.56	2.30E-01
cg2527	*dcp*, probable peptidyl-dipeptidase A protein	26.32	2.33E-01
cg0563	*rplK*, 50S ribosomal protein L11	26.21	2.56E-01
cg0610	*rplE*, 50S ribosomal protein L5	26.15	2.32E-01
cg1776	*tal*, transaldolase	26.12	2.61E-01
cg2127	hypothetical protein cg2127	25.97	2.53E-01
cg1761	*sufS*, Fe-S cluster assembly protein	25.25	2.05E-01
cg0914	*ftsE*, cell division ATP-binding protein	25.10	2.49E-01
cg1364	*atpF*, ATP synthase subunit B	24.78	2.55E-01
cg2470	secreted ABC transporter substrate-binding protein	24.58	2.20E-01
cg0204	*iolG*, putative oxidoreductase myo-inositol 2-dehydrogena	23.88	2.34E-01
cg2374	*murE*, UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	23.41	2.78E-01
cg1726	*mutB*, methylmalonyl-CoA mutase, small subunit	23.30	2.41E-01
cg0134	hydrolase, AMA/HIPO/HYUC family	22.54	2.52E-01
cg1698	*hisG*, ATP phosphoribosyltransferase	22.43	2.25E-01
cg1367	*atpG*, ATP synthase subunit C	21.51	1.86E-01
cg2139	*gluD*, glutamate permease	21.38	2.64E-01
cg1001	*mscL*, large conductance mechanosensitive channel	20.94	2.34E-01
cg0988	*rpsR*, 30S ribosomal protein S18	20.92	2.16E-01

cg2377	*mraW*, S-adenosyl-methyltransferase	20.90	2.70E-01
cg3306	*rplI*, 50S ribosomal protein L9	20.48	2.39E-01
cg2163	*dapB*, dihydrodipicolinate reductase	20.41	2.63E-01
cg1699	*hisE*, phosphoribosyl-ATP pyrophosphatase	19.83	2.32E-01
cg0915	*ftsX*, putative cell division protein	19.72	2.26E-01
cg0149	*panB*, 3-methyl-2-oxobutanoate hydroxymethyltransferase	19.41	2.78E-01
cg1842	putative secreted metalloprotease	19.41	2.38E-01
cg0199	*iolA*, myo-Inositol catabolism, aldehyde dehydrogenase	18.91	2.04E-01
cg0913	*prfB*, peptide chain release factor 2	18.30	2.79E-01
cg1479	*malP*, maltodextrin phosphorylase	17.78	7.26E-02
cg1813	*carB*, carbamoyl-phosphate synthase large subunit	17.61	2.18E-01
cg1873	*tesB2*, probable acyl-CoA thioesterase II protein	17.30	2.50E-01
cg2222	*rpsB*, 30S ribosomal protein S2	17.21	2.62E-01
cg2176	*infB*, translation initiation factor IF-2	17.21	2.30E-01
cg3396	membrane protease subunit, stomatin/prohibitin homologs	15.82	2.06E-01
cg3032	putative secreted protein	15.74	2.86E-01
cg0693	*groEL*, 60 KDA chaperonin (protein CPN60) (groel protein) C-terminal fragment	15.48	2.92E-01
cg0399	hypothetical protein cg0399	14.92	2.47E-01
cg2642	*benK1*, putative benzoate transport protein	14.62	2.24E-01
cg2153	similar to competence-and mitomycin-induced protein	13.44	2.80E-01
cg1837	holliday junction resolvase-like protein	13.42	2.46E-01
cg1075	*prsA*, ribose-phosphate pyrophosphokinase	13.18	2.31E-01
cg0609	*rplX*, 50S ribosomal protein L24	13.11	1.92E-01
cg0239	hypothetical protein cg0239	12.61	2.55E-01
cg3356	Na+/H+-dicarboxylate symporter	12.16	1.19E-01
cg2789	*nrdH*, putative glutaredoxin NRDH	11.96	3.09E-01
cg1052	*cmt3*, corynomycolyl transferase	11.85	2.82E-01
cg0691	*groEL'*, 60 KDA chaperonin (protein CPN60) (HSP60)-N-terminal fragment	11.71	2.99E-01
cg0957	*fas*-IB, fatty acid synthase	11.62	1.39E-01
cg3213	putative secreted protein	11.15	6.19E-02
cg0787	transcriptional regulator	10.89	2.43E-01
cg0581	*rpsL*, 30S ribosomal protein S12	10.84	1.06E-01
cg0582	*rpsG*, 30S ribosomal protein S7	10.83	9.88E-02
cg0413	*cmt1*, trehalose corynomycolyl transferase	10.54	2.64E-01
cg0296	*dnaZX*, DNA polymerase III subunits gamma and tau	10.34	2.92E-01
cg0791	*pyc*, pyruvate carboxylase	9.79	1.08E-01
cg3113	hypothetical protein cg3113	9.57	4.09E-01
cg1613	*sseA2*, rhodanese-related sulfurtransferase	9.50	2.97E-01
cg2249	*trmD*, tRNA (guanine-N(1)-)-methyltransferase	9.38	2.53E-01
cg0656	*rplQ*, 50S ribosomal protein L17	8.92	2.45E-01
cg0999	putative molybdenum cofactor biosynthesis protein	8.36	3.22E-01
cg0441	*lpd*, dihydrolipoamide dehydrogenase	8.06	2.44E-01
cg0267	*aroT*, aminotransferase, uses aromatic amino acids	7.91	3.08E-01
cg1436	*ilvN*, acetolactate synthase small subunit	7.82	1.98E-03
cg1725	*mutA*, methylmalonyl-CoA mutase, subunit	7.79	2.01E-01
cg2595	*rplU*, 50S ribosomal protein L21	7.59	2.38E-02
cg0998	trypsin-like serine protease	7.34	2.21E-01

cg1476	*thiC*, thiamine biosynthesis protein ThiC	6.92	6.09E-02
cg3189	hypothetical protein cg3189	6.64	1.44E-01
cg3013	hypothetical protein cg3013	6.19	3.26E-01
cg1332	putative secreted hydrolase	6.11	1.23E-01
cg2888	*phoR*, two component response regulator	6.07	7.89E-02
cg1579	putative secreted protein	5.74	1.73E-01
cg0755	*metY*, O-acetylhomoserine sulfhydrylase	5.74	3.43E-01
cg2586	*proA*, gamma-glutamyl phosphate reductase	5.69	3.11E-01
cg2177	predicted nucleic-acid-binding protein implicated in transcription termination	5.50	2.09E-01
cg3430	hypothetical protein cg3430	5.32	3.56E-01
cg1731	membrane protein implicated in regulation of membrane protease activity	5.16	3.55E-01
cg0700	*guaB3*, inositol-5-monophosphate dehydrogenase	5.15	3.15E-01
cg0628	*rpsH*, 30S ribosomal protein S8	5.07	7.40E-02
cg1053	putative TetR-family transcriptional regulator	4.81	2.37E-01
cg1531	*rpsA*, 30S ribosomal protein S1	4.72	1.98E-01
cg2974	*lysS*, lysyl-tRNA synthetase	4.72	2.29E-01
cg3308	*rpsF*, 30S ribosomal protein S6	4.65	7.90E-02
cg0990	*rpmG*, 50S ribosomal protein L33	4.65	4.02E-02
cg1810	*gmk*, guanylate kinase	4.61	2.57E-01
cg0878	*whcE*, positive role in survival under (heat and oxidative) stress	4.57	6.90E-02
cg2593	putative secreted or membrane protein	4.57	3.78E-01
cg1109	*porB*, anion-specific porin precursor	4.54	3.51E-01
cg3119	*cysJ*, probable sulfite reductase (flavoprotein)	4.50	4.96E-05
cg0991	*rpmB*, 50S ribosomal protein L28	4.45	3.39E-01
cg0653	*rpsK*, 30S ribosomal protein S11	4.34	5.52E-03
cg2137	*gluB*, glutamate secreted binding protein	4.16	3.45E-01
cg2799	*pknE*, putative secreted protein	4.14	2.93E-01
cg0403	*rmlB1*, dTDP-glucose 4,6-dehydratase	4.14	1.08E-01
cg3346	*leuS*, leucyl-tRNA synthetase	4.01	4.45E-01
cg3342	putative secreted protein	4.00	3.16E-01
cg2138	*gluC*, glutamate permease	3.98	2.84E-01
cg0812	*dtsR1*, acetyl/propionyl-CoA carboxylase beta chain	3.95	4.10E-01
cg1248	GTPase involved in stress response	3.94	4.59E-01
cg1435	*ilvB*, acetolactate synthase I large subunit	3.91	1.03E-01
cg1403	*gatC*, aspartyl/glutamyl-tRNA amidotransferase subunit C	3.87	4.54E-01
cg0060	*pbpA*, D-alanyl-D-alanine carboxypeptidase	3.84	4.66E-01
cg3021	hypothetical protein cg3021	3.83	4.20E-01
cg3307	*ssb*, single-strand DNA-binding protein	3.83	2.01E-01
cg1656	*ndh*, NADH dehydrogenase	3.77	4.74E-01
cg0373	*topA*, DNA topoisomerase I	3.75	4.80E-01
cg0491	hypothetical protein cg0491	3.73	4.63E-01
cg0400	*adhC*, alcohol dehydrogenase, class C	3.71	4.72E-01
cg0391	*rmlB2*, putative dTDP-glucose 4,6-dehydratase	3.66	4.87E-01
cg0603	*rpmC*, 50S ribosomal protein L29	3.65	4.83E-01
cg1432	*ilvD*, dihydroxy-acid dehydratase	3.62	4.95E-01
cg1814	*carA*, carbamoyl-phosphate synthase small subunit	3.60	4.98E-01

cg1811	*ihf*, putative integration host factor cIHF	3.58	4.89E-01
cg2429	*glnA*, glutamine synthetase I	3.51	3.01E-01
cg2175	*rbfA*, ribosome-binding factor A	3.49	4.76E-01
cg1763	*sufD*, Fe-S cluster assembly membrane protein	3.43	4.57E-01
cg0654	*rpsD*, 30S ribosomal protein S4	3.43	2.60E-01
cg2949	putative secreted protein	3.36	3.84E-01
cg3186	*cmt2*, trehalose corynomycolyl transferase	3.30	1.54E-01
cg0602	*rplP*, 50S ribosomal protein L16	3.25	4.08E-01
cg1836	secreted solute-binding protein, aminodeoxychorismate lyase-like	3.24	2.02E-01
cg1705	*arsB1*, arsenite permease	3.23	3.95E-01
cg1270	probable O-methyltransferase	3.22	3.15E-01
cg3315	bacterial regulatory protein, MarR family	3.22	2.80E-01
cg0690	*groES*, chaperonin 10 Kd subunit	3.19	4.29E-02
cg3031	hypothetical protein cg3031	3.17	3.98E-01
cg0401	*rmlA1*, TDP-glucose pyrophosphorylase	3.16	3.27E-01
cg0955	secreted protein	3.10	1.55E-03
cg0062	*ppp*, protein phosphatase	3.10	3.67E-01
cg0599	*rpsS*, 30S ribosomal protein S19	3.05	3.43E-01
cg0300	hypothetical tripeptide synthase involved in murein formation	3.04	5.73E-04
cg1437	*ilvC*, ketol-acid reductoisomerase	3.03	8.88E-02
cg2828	hypothetical protein cg2828	2.92	2.19E-01
cg1816	*pyrB*, aspartate carbamoyltransferase catalytic subunit	2.92	2.56E-01
cg2850	hypothetical protein cg2850	2.90	1.08E-02
cg0446	*sdhA*, succinate dehydrogenase	2.89	1.89E-01
cg2865	*purS*, phosphoribosylformylglycinamidine synthase	2.88	3.97E-05
cg2071	*int2'*, putative phage integrase (N-terminal fragment)	2.86	2.40E-01
cg0600	*rplV*, 50S ribosomal protein L22	2.86	3.14E-01
cg0061	*rodA*, putative FTSW/RODA/SPOVE family cell cycle protein	2.84	1.74E-01
cg2103	*dtxR*, diphtheria toxin repressor	2.83	9.11E-02
cg0492	extremely conserved possible DNA-binding protein	2.82	2.61E-01
cg3260	hypothetical protein cg3260	2.82	2.64E-01
cg0447	*sdhB*, succinate dehydrogenase	2.78	9.08E-04
cg1903	ABC-type multidrug transport system, ATPase component	2.76	2.58E-01
cg0307	*asd*, aspartate-semialdehyde dehydrogenase	2.75	7.13E-02
cg1789	*tpi*, triosephosphate isomerase	2.73	1.40E-01
cg1612	acetyltransferase	2.73	1.53E-01
cg0067	*gabD3*, succinate-semialdehyde dehydrogenase (NADP+)	2.72	9.39E-02
cg1825	*efp*, elongation factor p	2.71	9.11E-02
cg1121	permease of the major facilitator superfamily	2.70	3.76E-03
cg1243	secreted trypsin-like serine protease, contain C-terminal PDZ domain	2.69	1.04E-08
cg0786	*upp*, uracil phosphoribosyltransferase	2.67	1.55E-01
cg2430	hypothetical protein cg2430	2.64	1.72E-01
cg3424	*cwlM*, N-acetymuramyl-L-alanine amidase	2.63	1.98E-01
cg1577	putative secreted hydrolase	2.62	2.05E-01
cg0306	*lysC*, aspartate kinase	2.58	1.83E-01

cg0594	*rplC*, 50S ribosomal protein L3	2.54	1.39E-02
cg1044	hypothetical protein cg1044	2.50	3.60E-02
cg3050	acyltransferase	2.50	2.07E-02
cg1819	nucleoside-diphosphate sugar epimerase (SulA family)	2.47	2.41E-02
cg3008	*porA*, main cell wall channel protein	2.47	9.34E-02
cg1794	hypothetical protein cg1794	2.45	2.77E-10
cg0703	*guaA*, bifunctional GMP synthase/glutamine amidotransferase protein	2.44	1.03E-01
cg0754	*metA*, homoserine O-acetyltransferase	2.41	1.98E-04
cg2124	hypothetical protein cg2124	2.39	3.63E-15
cg2523	*malQ*, 4-alpha-glucanotransferase	2.37	3.91E-02
cg3014	hypothetical protein cg3014	2.37	2.66E-02
cg0715	secreted protein	2.36	3.40E-15
cg1537	*ptsG*, glucose-specific enzyme II BC component of PTS	2.33	8.27E-02
cg0350	*glxR*, cAMP-dependent transcriptional regulator	2.33	1.38E-17
cg1420	*gatB*, aspartyl/glutamyl-tRNA amidotransferase subunit B	2.32	4.66E-02
cg1817	*pyrR*, pyrimidine regulatory protein PyrR	2.32	6.07E-18
cg2703	sugar permease	2.31	8.36E-13
cg2830	*pduO*, adenosylcobalamin-dependent diol dehydratase gamma	2.30	5.42E-03
cg2409	*ctaC*, cytochrome C oxidase chain II	2.29	1.27E-10
cg2863	*purQ*, phosphoribosylformylglycinamidine synthase subunit	2.24	3.78E-04
cg1774	*tkt*, transketolase	2.22	6.57E-12
cg2366	*ftsZ*, cell division protein FtsZ	2.15	3.84E-20
cg3009	*porH*, main cell wall channel protein	1.29	2.72E-01
cg1671	putative membrane-associated GTPase	1.22	2.28E-01
cg2792	*nadE*, NAD(+) synthetase	1.15	2.04E-01
cg1514	secreted protein	1.00	1.87E-01
cg1345	*narK*, putative nitrate/nitrite transporter	0.43	3.23E-03
cg0175	secreted protein, signal peptide	0.43	7.19E-03
cg2572	hypothetical protein cg2572	0.38	3.08E-02
cg1045	hypothetical protein cg1045	0.38	2.47E-02
cg0796	*prpD1*, propionate catabolic protein PRPD	0.36	2.82E-02
cg1392	transcriptional regulator, CRO/CI family	0.33	3.80E-02
cg3100	*dnaK*, molecular chaperone Dnak	0.33	3.73E-08
cg1543	iunH3, inosine-uridine preferring nucleoside hydrolase	0.32	4.65E-02
cg0798	*prpC1*, citrate synthase	0.32	4.09E-05
cg3052	putative secreted protein	0.31	1.74E-18
cg0797	*prpB1*, probable methylisocitric acid lyase	0.28	4.89E-02
cg3051	putative secreted protein	0.24	2.57E-02
cg1546	*rbsK1*, putative ribokinase protein	0.22	5.31E-02
cg2999	putative ferredoxin reductase	0.21	1.93E-02
cg2837	*sucC*, succinyl-CoA synthetase subunit beta	0.00	1.05E-01

[a)] Two biological replicates were performed. To evaluate and filter the generated data, the signal-to-noise ratio for the individual data points was set to >5 and only genes whose ratio was changed at least two-fold were considered. Listed are all genes which were regulated in both performed experiments with their average ratio of medians and p-values. Only genes with a p-value <0.05 were considered as significant.

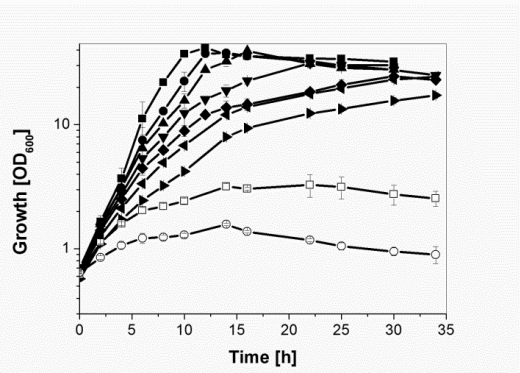

Figure S1 Growth inhibition of *C. glutamicum* wild type in CGXII minimal medium supplemented with 111 mM glucose and different methanol concentrations ranging from 0.05 M to 3 M (0.05 M (●), 0.1 M (▲), 0.2 M (▼), 0.7 M (♦), 1 M (◄), 1.3 M (►), 1.5 M (□), 3 M (○)). As control, *C. glutamicum* wild type was grown in CGXII-minimal medium with 111 mM glucose without addition of methanol (■).

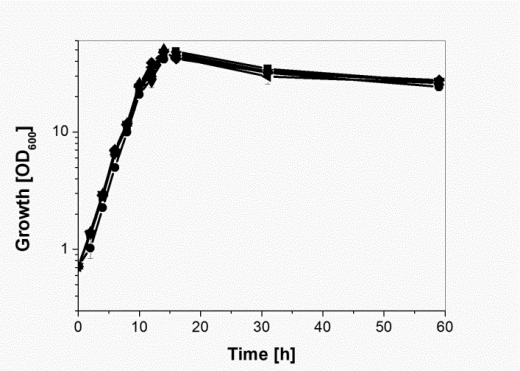

Figure S2 Growth of the *C. glutamicum* strain wild type (■), Δ*adhA* (●), Δ*adhC* (▲), Δ*adhE* (▼), Δcg2714 (♦), and Δcg0273 (◄) in CGXII minimal medium supplemented with 111 mM glucose and 120 mM methanol. Two to four biological replicates were performed. Mean values are shown.

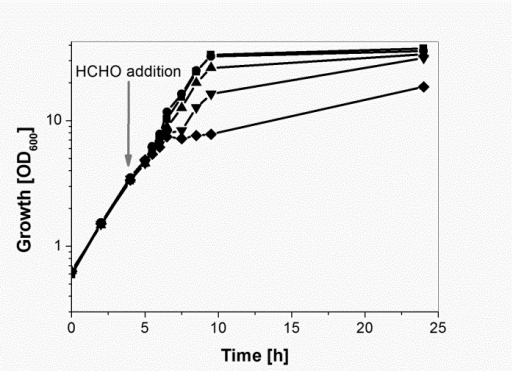

Figure S3 Influence of formaldehyde on growth of *C. glutamicum* wild type. After 4 h of cultivation, 1 mM (●), 2 mM (▲), 3 mM (▼) or 4 mM (♦) formaldehyde was added to the culture. As control, no formaldehyde was added to the medium (■).

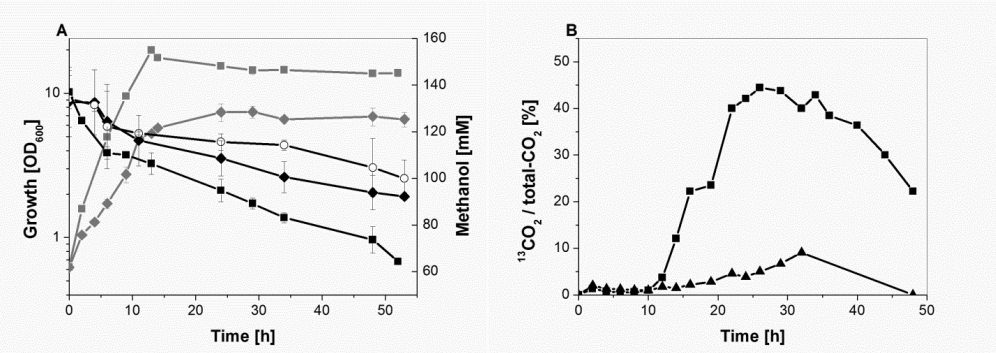

Figure S4 (A) Methanol oxidation (♦) and growth (♦) of *C. glutamicum* Δ*ramA* in comparison to methanol oxidation (■) and growth (■) of *C. glutamicum* wild type in CGXII minimal medium supplemented with 56 mM glucose and 120 mM methanol. Three independent experiments were performed, always accompanied by a methanol evaporation control (○). (B) $^{13}CO_2$-formation as $^{13}CO_2$/total CO_2 ratio of *C. glutamicum* Δ*ramA* (▲) and *C. glutamicum* wild type (■) during growth experiments in bioreactors in the presence of 100 mM ^{13}C-labelled methanol.

6.3 Supplementary material "Engineering of *Corynebacterium glutamicum* towards methanol utilization"

Abbreviations

Amino acids are presented according to their 3-letter standard abbreviations; AKG, ◎-ketoglutarate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUC, succinate; 23PG, 2-/3-phosphoglycerate; 6PG, 6-phosphogluconate; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; S7P: sedoheptulose-7-phosphate.

Plasmid construction

The oligonucleotides used for gene amplification by PCR are listed in Table S1. The *adhA* gene (cg3107) was amplified from the genomic DNA of *C. glutamicum* using the oligonucleotides P_*adhA*_fw_*Sal*I and P_*adhA*_rev_*Xba*I. The PCR product was restricted with *Sal*I/ *Xba*I and cloned into pVWEx2 resulting in pVWEx2-Cg*adhA*. For construction of pVWEx2-Bm(*mdh-act*) and pVWEx2-Bm(*mdh3-act*) the *mdh* gene was excised with *Pst*I/ *Sal*I, the *mdh3* gene with *Nde*I/ *Sal*I and the *act* gene with *Sal*I/ *Xba*I from the provided standard Life Technologies vectors and sub-cloned into the vector pVWEx2.

Construction of pEKEx2-Bs(*hps-phi*) was performed via amplification of *yckG* (from now on designated as *hps*) and *yckF* (from now on designated as *phi*) from the genomic DNA of *Bacillus subtilis* 168 by PCR using the oligonucleotides P_*yckG*-fw-*Sal*I and P_*yckF*-rev-*BamH*I. The amplified PCR product was restricted with *Sal*I/ *BamH*I and cloned into the vector pEKEx2. The *hps-phi* operon of *Mycobacterium gastri* was excised from the standard vector provided by Life Technologies and sub-cloned into the vector pEKEx2 using the restriction sites *Pst*I and *Kpn*I. The resulting *C. glutamicum* expression vector was called pEKEx2-Mg(*hps-phi*).

For expression of genes under the control of the constitutive promoter P*tuf* [as realized in the vectors pVWEx2-P*tuf*-Bm(*mdh-act*), pVWEx2-P*tuf*-Bm(*mdh3-act*) and pEKEx2-P*tuf*-Bs(*hps-phi*)], the vectors pVWEx2-Bm(*mdh-act*) and pEKEx2-Bs(*hps-phi*) were restricted with *Sal*I and *Apa*I, and the vector pVWEx2-Bm(*mdh3-act*) with *Apa*I and *Nde*I, which resulted in the excision of the P*tac* promoter and *lacIQ*. Subsequently, the P*tuf* was PCR-amplified and cloned into the restricted vectors. The promoter was amplified using the following oligonucleotides:

Amplification of P*tuf* for cloning in front of Bm(*mdh3-act*): P*tuf*_fw_*Apa*I and P*tuf*_rev_*Nde*I and for cloning in front of Bm(*mdh-act*) and Bs(*hps-phi*): P_P*tuf*_fw_*Apa*I and P_P*tuf*_rev_*Sal*I.

Table S1 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′) and properties [a]
Amplification of *C. glutamicum adhA* gene	
P_ *adhA*-fw-*Sal*I	AGC GTC GAC AAG GAG ATA TAC ATA TGA CCA CTG (*Sal*I)
P_ *adhA*-rev-*Xba*I	AGC TCT AGA TTA GAA ACG AAT CGC CAC ACG (*Xba*I)
Amplification of the promoter P*tuf*	
P_P*tuf*-fw-*Apa*I	CAG TGG GCC CCC ACA GGG TAG CTG GTA GTT TG (*Apa*I)
P_P*tuf*-rev-*Sal*I	CAG TGT CGA CGG ACT TCG TGG TGG CTA CGA C (*Sal*I)
P_P*tuf*-rev-*Nde*I	CAG TCA TAT GGG ACT TCG TGG TGG CTA CGA C (*Nde*I)
Amplification of *B. subtilis yckF* and *yckG* genes	
P_*yckG*-fw-*Sal*I	TTT AAG TCG ACG AAA GGA GGA TAG ATA TGG AAT TAC AGC TTG CAT TAG A (*Sal*I)
P_*yckF*-rev-*BamH*I	AAT TTG GAT CCC TAT TCA AGG TTT GCG TGG TG (*BamH*I)
Sequencing of *adhA, yckF* and *yckG* as well as P*tuf* in pVWEx2 and pEKEx2	
P_pEKEx-for2	CGG CGT TTC ACT TCT GAG TTC GGC
P_pEKEx-rev2	GAT ATG ACC ATG ATT ACG CCA AGC
P_P*tuf*-seq-fw	AGC TGT CTT CGG TAT CGT CGT ATC

[a] Recognition sites for the indicated restriction enzymes are underlined.

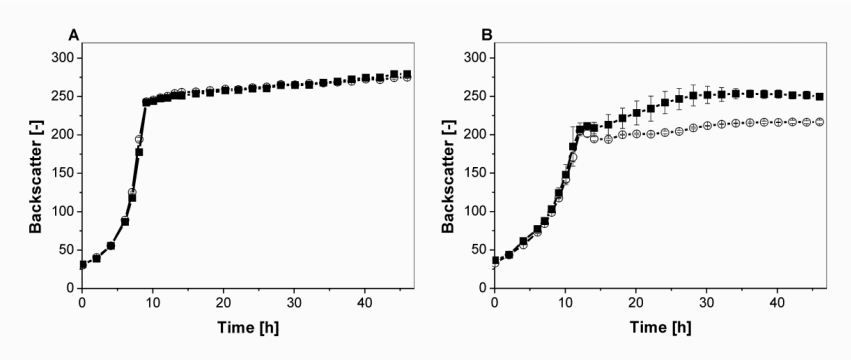

Figure S1 Growth of recombinant *C. glutamicum* strains in CGXII minimal medium supplemented with 55 mM glucose (**A**) and in medium supplemented with 55 mM glucose and 120 mM methanol (**B**). Strains: *C. glutamicum* Bm(*mdh-act*) (■) and *C. glutamicum* pVWEx2 (○). Induction of gene expression was performed with 1.5 mM IPTG. Monitoring of the growth was performed in 48-well FlowerPlates using a BioLectorBasic.

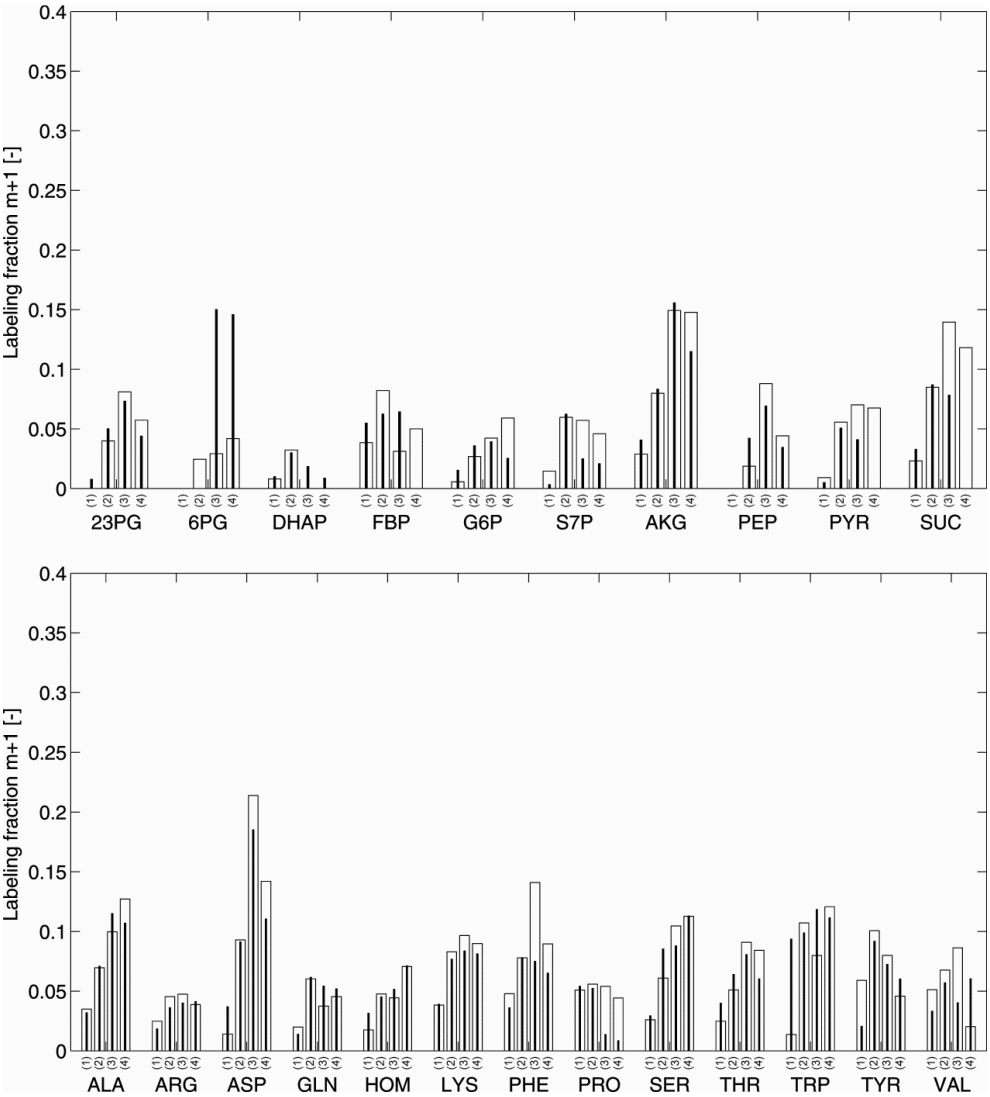

Figure S2 Labelling fractions of the m+1 mass isotopomers of selected intracellular metabolites and amino acids for the strain *C. glutamicum* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*). Two independent batch reactors were run with CGXII minimal medium supplemented with 55 mM glucose and 120 mM ^{13}C-labelled methanol. Quenching of metabolic activity as well as intracellular metabolic analysis was performed in the exponential phase (**1**), in the transition to the stationary growth phase/second growth phase (**2**) as well as 12h (**3**) and 24h (**4**) after reaching the stationary phase. The measured values were corrected for the natural ^{13}C-abundance.

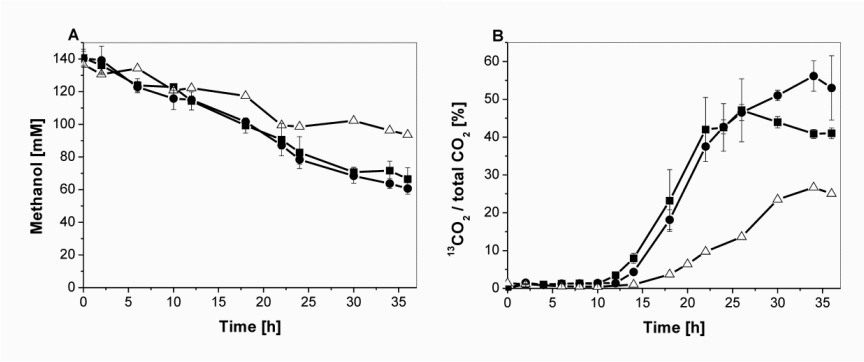

Figure S3 (**A**) Methanol consumption and (**B**) $^{13}CO_2$ formation given as the $^{13}CO_2$/total CO_2 ratio of *C. glutamicum* Δ*ald*Δ*adhE* strains expressing either P*tuf*-Bm(*mdh-act*) and P*tuf*-Bs(*hps-phi*) (■) or P*tuf*-Bm(*mdh3-act*) and P*tuf*-Bs(*hps-phi*) (●) and of the negative control *C. glutamicum* Δ*ald*Δ*adhE* pVWEx2 pEKEx2 (Δ) during bioreactor cultivations in CGXII minimal medium supplemented with 55 mM glucose and 120 mM ^{13}C-labeled methanol. Two independent experiments were performed and mean values are shown.

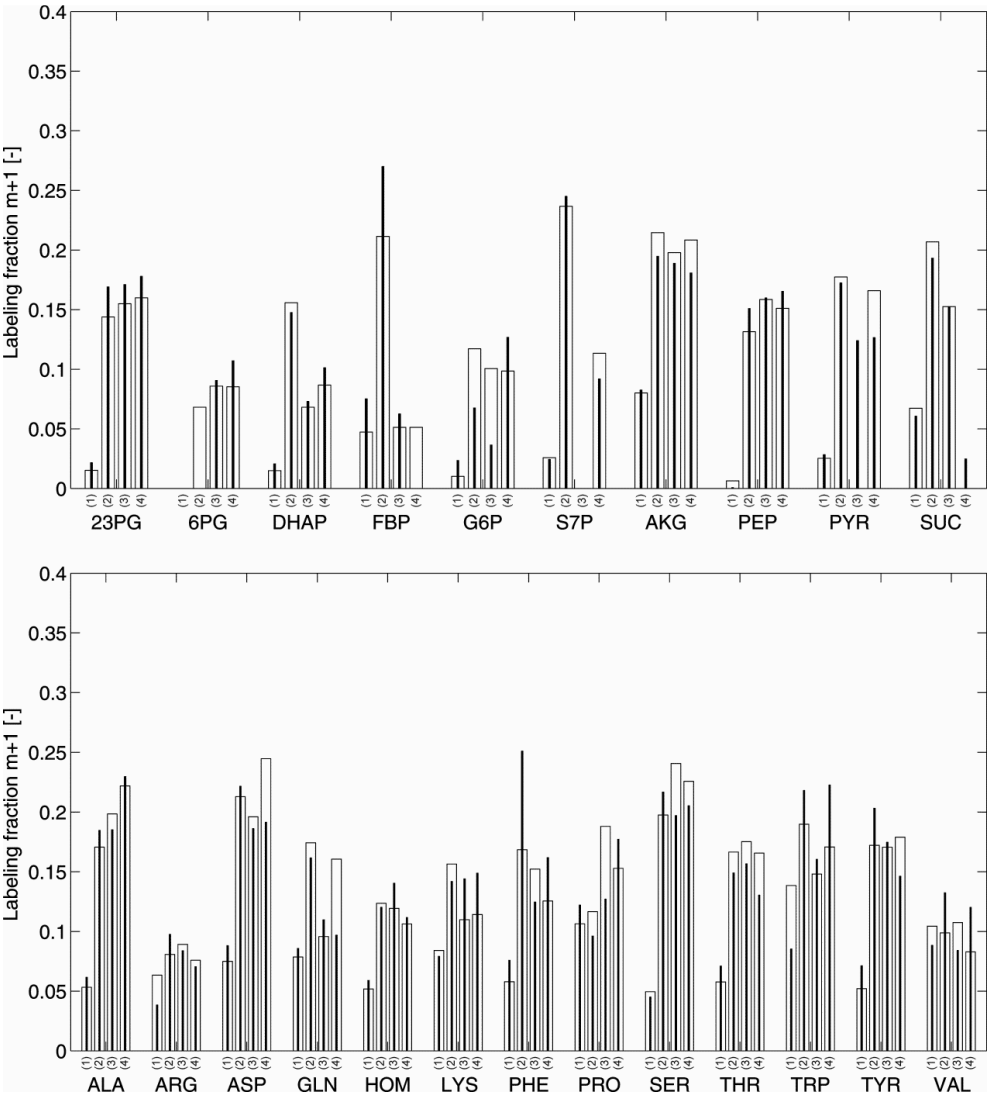

Figure S4 Labelling fractions of the m+1 mass isotopomers of selected intracellular metabolites and amino acids for the strain *C. glutamicum* Δ*ald* Δ*adhE* P*tuf*-Bm(*mdh-act*) P*tuf*-Bs(*hps-phi*). Two independent batch reactors were run with CGXII minimal medium supplemented with 55 mM glucose and 120 mM ^{13}C-labelled methanol. Quenching of metabolic activity as well as intracellular metabolic analysis was performed in the exponential phase (**1**), in the transition to the stationary growth phase/second growth phase (**2**) as well as 12h (**3**) and 24h (**4**) after reaching the stationary phase. The measured values were corrected for the natural ^{13}C-abundance.

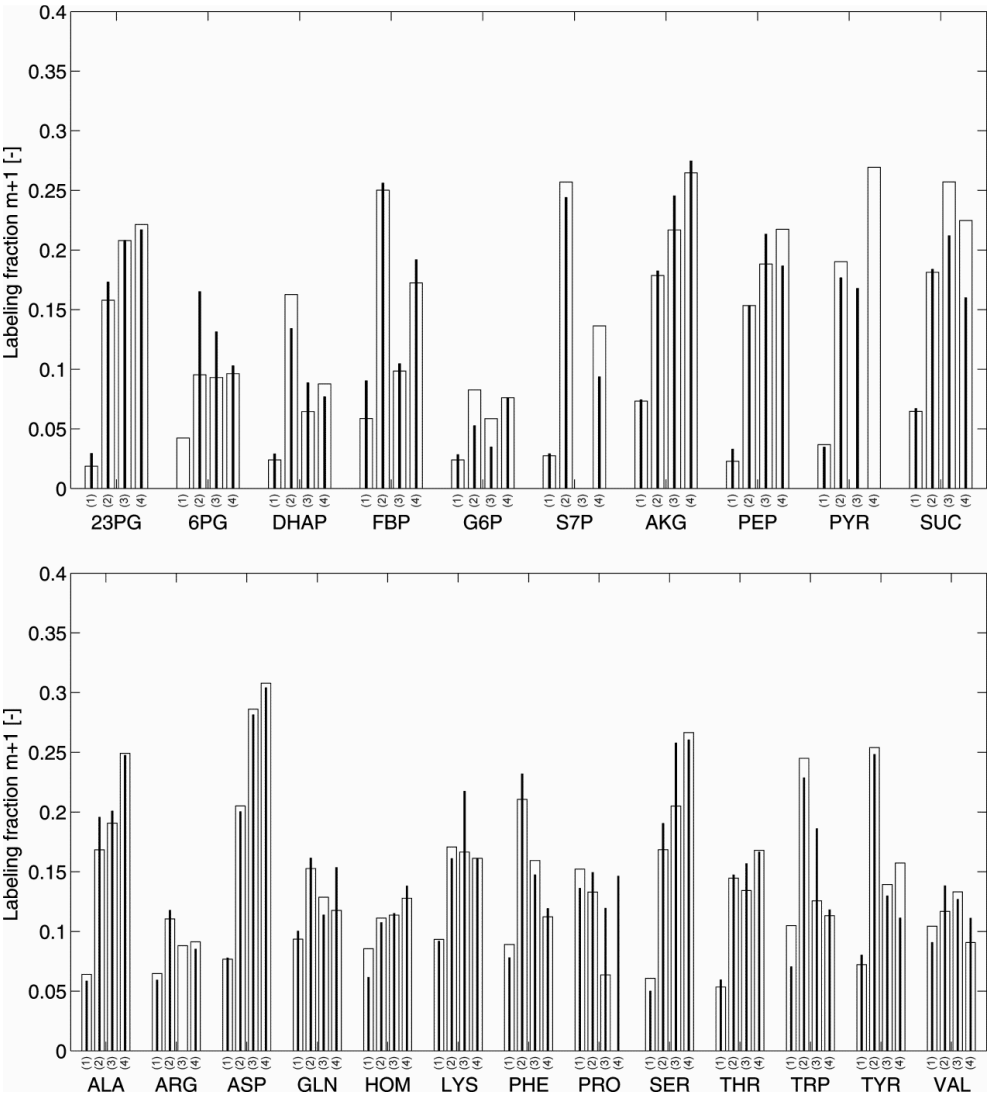

Figure S5 Labelling fractions of the m+1 mass isotopomers of selected intracellular metabolites and amino acids for the strain *C. glutamicum* Δ*ald* Δ*adhE* P*tuf*-Bm(*mdh3-act*) P*tuf*-Bs(*hps-phi*). Two independent batch reactors were run with CGXII minimal medium supplemented with 55 mM glucose and 120 mM ^{13}C-labelled methanol. Quenching of metabolic activity as well as intracellular metabolic analysis was performed in the exponential phase (**1**), in the transition to the stationary growth phase/second growth phase (**2**) as well as 12h (**3**) and 24h (**4**) after reaching the stationary phase. The measured values were corrected for the natural ^{13}C-abundance.

6.4 Supplementary references

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Ikeda M, Nakagawa S (2003). The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes**.** *Appl Microbiol Biotechnol* **62**, 99–109**.**

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegrabe I, Tauch A (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* **104**, 5–25.

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Erklärung

Die hier vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Sabrina Witthoff

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